

Slow, Latent, and Temperate
VIRUS INFECTIONS

Slow Virus Infections

Slow, Latent, and Temperate VIRUS INFECTIONS

Edited by

D. CARLETON GAJDUSEK
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Foreword

The National Institute of Neurological Diseases and Blindness has a long term commitment to the study of multiple sclerosis, amyotrophic lateral sclerosis and other chronic degenerative disorders of the central nervous system, the etiology of which still remains obscure. The research projects in our Institute, involving the remote and isolated intense foci of chronic central nervous system disease, namely, the study of kuru in New Guinea and of motor neuron disease foci in Guam and West New Guinea, have served to draw our attention once again to the possibility that an infectious agent may be involved in the pathogenesis of these disorders. With this renewed inquiry, we are forced also to reconsider the common sporadic subacute degenerative disorders of the nervous system, including multiple sclerosis, as they are seen in America and elsewhere in the world. The similarity of certain of these processes to scrapie has further revived this interest. Much of the hope which scientists have for finding a transmissible agent in at least some of these disorders rests upon the advances which veterinarians and virologists have made in attributing this and other analogous diseases in domestic animals to viral agents. This approach in no way excludes our continued interests in possible co-involvement of infection with a viral agent with disturbances in the immune mechanism—perhaps, with auto-immune hypersensitivity or genetic determination of susceptibility.

During the past few years, under the primary impetus of kuru investigations in New Guinea, this Institute has established, under Dr. D. Carleton Gajdusek's direction, a laboratory for the study of slow, latent, and temperate virus infections. The investigators in this section have felt that it was timely, in fact, urgently desirable, that a working discussion meeting be held at which investigators in immunology, veterinary virology of slow infections, and cancer virology, as well as other medical virologists, could present and discuss their contributions informally for an extensive exchange of ideas. The meetings, held in December of 1964 at the NIH, were, I believe, considered by all participants to have been very successful and valuable to them. Therefore, the Institute has undertaken the publication of a more formal presentation of the contributions of the participants in the form of this Monograph.

We were happy to be hosts to the many investigators who assembled here and were particularly grateful to them for their additional efforts after the meetings in preparing, revising, and amplifying their contributions for this publication.

RICHARD L. MASLAND, M.D.,
*Director, National Institute of
Neurological Diseases and Blindness.*

Workshop and Symposium
on
Slow, Latent, and Temperate Virus Infections

Held at the
National Institutes of Health, Bethesda, Maryland

December 7, 8, and 9, 1964

Sponsored by the
National Institute of Neurological Diseases and Blindness
National Institutes of Health, Bethesda, Maryland

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Acknowledgments

The Workshop-Symposium was made possible through the continued encouragement, enthusiastic interest, and ample support given by Dr. Richard L. Masland, Director of the sponsoring National Institute of Neurological Diseases and Blindness.

We thank the participants of the Workshop-Symposium whose presence assured its success and whose work provided the substance of this monograph. The temerity of our small laboratory to attempt to conduct such a large workshop has rested largely upon the willingness of the persons who gave so much of their time and efforts preceding, during, and following the Workshop sessions.

Preface

After microbiology had given solution to the etiology of most acute infections of the central nervous system and after fungi and bacteria had been incriminated in important chronic disorders of the nervous system such as torula and tuberculous meningitis, we have been left, in neurology, with a wide range of subacute and chronic affections of the central nervous systems of unknown etiology. Some of these diseases, still listed as idiopathic, are among the most prevalent afflictions of the central nervous system. Many others with familial patterns of occurrence do not yet have their basic pathogenesis or underlying metabolic defect elucidated, although we tend to think of them as genetically mediated. If any of these diseases fall into the category of slow, latent, or temperate virus infections—if even a single one of these many syndromes of unknown etiology can be traced to a virus or virus-gene interaction—we shall win a significant advance in our understanding of diseases of the human brain.

The founders of the virological discipline were early concerned with the latency and masking of herpes simplex virus and the recurrence of herpetic infections, with the long persistence of many pox-virus infections, and with the long incubation period (sometimes approaching one year) in rabies. At the dawn of virology latency, masking, and slowness were prominent in the minds of investigators in many classical studies. Furthermore, early immunologists from Paul Ehrlich and Metchnikoff on considered the possibility that disturbances of the immune mechanism associated with virus infections might produce autoaggressive phenomena and that carrier states and persistent infections might result from non-reactivity of the immune mechanism.

However, in the current era of medical virology, we have been somewhat spell-bound by success in elucidating the pathogenesis of acute viral infections and in producing vaccines to immunize against them. The old problem has slumbered restlessly in the medical clinic until the past decade, although this is not a new field of inquiry for insect and plant virologists or more recently for cancer investigators. It is now time that neurologists too renewed their attack on their age-old, still unsolved, problems, bringing the modern methods of immunology and microbiology to bear once again on these mysteries, with the powerful new techniques of tissue culture, immunofluorescence, and electronmicroscopy to aid them and the new understanding of temperate infections and virus-gene interaction and of the immune mechanism to direct their thinking. This Workshop-Symposium is an attempt to bring together those actively engaged in trying to apply virological and immunological techniques to the problems of the subacute and chronic idiopathic disorders of the nervous system in animals and in man.

This meeting was planned less as a formal symposium or international meeting, than as an informal gathering of workers actively engaged in laboratory work with viruses which illustrate properties of latency, masking, slowness or temperateness. We particularly wished to bring the medical virologists together with those working in veterinary virology, who have made such important basic contributions to this field. Although emphasis was to be on subacute and chronic neurological disorders

of unknown etiology which might be virus-evoked, the advances in the study of latency and masking of viruses causing other disorders, particularly the study of tumor viruses, were much on our minds. The successes in tumor virus studies have done much to turn our thinking toward possible virus etiology in a large number of idiopathic heredofamilial degenerative disorders of the central nervous system and the subacute and chronic encephalitides.

We planned for as much discussion time as time for formal presentation of papers. We were anxious that the meeting be small enough to gather all participants around a conference table for informal discussion and exchange of data on methods and procedures and approaches. We were more concerned with the actual details of experiments in progress than on theoretical approach. However, the scope of the conference was so large and the interest in it so great that our list of participants grew beyond the two dozen we had originally envisaged; it was finally difficult to find a table large enough to accommodate everyone. Nevertheless, we were adamant in keeping the meeting moderately informal—a workshop, rather than a series of formal lectures. Thus, we restricted invitations rather arbitrarily to those investigators whom we knew were working intimately and actively in this field.

Contributors have been advised to alter, change, and augment their contributions which appear in this publication beyond the form in which they were presented and discussed at the meeting, in order that the current volume on this newly developing field be as up-to-date and comprehensive as possible.

We had hoped to include with this monograph a good deal of the discussion. However, since many of the contributions were written, and others rewritten, after the workshop disbanded, they have thereby answered many of the questions and disposed of many of the problems brought up in discussion. There have also been technical difficulties with the transcriptions and problems with the editing of the lengthy discussions to publishable form in keeping with the altered papers, but we have managed to preserve some of the discussion which gave so much life to the workshop. It is our belief that the expanded and revised forms of the formal contributions have themselves rejuvenated this compendium, and it is our hope that the evolution of ideas that accompanied and followed the meeting has, in a sense, made much of the discussion vestigial.

In conclusion, we wish to make some specific acknowledgments of assistance rendered in organizing the Workshop-Symposium: Dr. Michael Alpers; Mrs. Marion Poms, who served as general secretary; Mr. John Smart, Mr. William Tolson, and Mrs. Toby Zinnecker, who handled all the many administrative problems of space, travel, and accommodations; Mr. Mint Basnight, Dr. Paul Brown, Mrs. Flora Feld, Mr. Michael Nicholson, Miss Nancy Rogers, and Miss Judith Shaw of our laboratory for their assistance during the sessions; and the members of the technical staff of our Patuxent laboratory whose efforts during the long term studies of chronic viral diseases have made possible our entry into this patience-trying field: Mr. Alfred Bacote, Mrs. Helene Gilbert, Mr. Michael Sulima and Mr. Edward van Steinberg.

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Human Nervous System Diseases of Suspected Viral Etiology

Chairman

HILARY KOPROWSKI

Kuru in New Guinea and the Origin of the NINDB Study of Slow, Latent, and Temperate Virus Infections of the Nervous System of Man

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In 1957, Dr. Vincent Zigas and I first described the rapidly fatal disease, kuru, a strange new subacute, familial, degenerative disease of the central nervous system (characterized by cerebellar ataxia and trembling) and restricted in occurrence to some 12,000 native Highland New Guineans of the Fore linguistic group, and to their immediate neighbors with whom they intermarry, and among whom it accounted for over half of all deaths (1, 2).

On first seeing kuru, we had suspected it to be a viral meningoencephalitis, only to find very little in the clinical picture, laboratory findings, or epidemiology to support such a suspicion, and nothing in the neuropathology to suggest acute infection. The epidemiological pattern of kuru occurrence suggested some genetic determinant of disease expression and this was supported by the restriction of the disease in peripheral areas to those individuals genetically related to the population in the center of the region. However, our failure to find any febrile response, any cerebrospinal fluid pleocytosis, or elevated protein content, a regularly antecedent acute febrile disease, or sufficient perivascular cuffing or other neuropathologic reactions suggestive of infection led us to discount the possibility of acute infectious etiology. Further, we were unable to demonstrate any contact infections in people living in close association to kuru victims throughout the course of their disease. We had early considered association of the disease with extensive cannibalism, but soon dismissed this as unlikely when cases of the disease were encountered in individuals whom we did not believe had engaged in the ritual cannibalistic consumption of diseased relatives, the prevailing practice in the region. The hypothesis that the disease might be an

autosensitization reaction, perhaps provoked by early sensitization to human brain through cannibalism in infancy or early childhood, likewise was not borne out either by neuropathology or by the search for auto-immune antibodies to brain antigen in serum specimens.

Finally, the hypothesis that the disease might be a late postinfectious phenomenon akin to the mysterious von Economo's encephalitis of 1918 in Europe was hardly tenable, since, as mentioned, we could establish no regularly antecedent infection. The contention that the antecedent infection might be inapparent was disturbingly difficult to investigate and could only be tested seroepidemiologically, but no consistent pattern of antibody to known infectious agents was found in sera from kuru patients (3).

In 1959, Hadlow (4) brought to our attention the close similarities between the neuropathology, clinical symptoms, and epidemiology of kuru and of scrapie in sheep, a central nervous system degeneration known to be caused by a slow virus infection, susceptibility to which is genetically determined (5, 6). Infection had before this seemed a very unlikely etiologic possibility for kuru. Now we were forced to reconsider the problem in the light of slow virus infections of the nervous system familiar to the veterinary virologists, of which scrapie and visna were the best elucidated examples.

Should kuru and the vast majority of subacute and chronic degenerative diseases of the central nervous system of man be caused by viral agents with prolonged incubation periods or very slow initiation to pathologic processes, such as occur in infections of sheep with either the visna or scrapie virus, all previous attempts at isolation of transmissible agents from

the human diseases would naturally have been unsuccessful, for, to our knowledge, no investigators had maintained their inoculated experimental animals under observation for periods as long as 5 years, as may at times be necessary with scrapie. In fact, rarely did virologists attempting virus isolation keep their experimentally inoculated animals for periods as long as 1 year; more usually the animals were disposed of within a few months, at the most. Furthermore, as Hadlow (4) and Sigurdsson, Pálsson, and van Bogaert (7) pointed out, neither in scrapie nor visna was there the neuropathologic picture usually seen in viral infections of the nervous system; both diseases tended to run an afebrile course, and in scrapie there was no cerebrospinal fluid pleocytosis or increase in protein content. It was, thus, necessary to acknowledge that our earlier attempts to isolate an agent from kuru brain material by inoculation of small laboratory animals, tissue cultures, and embryonated chick eggs, had been woefully inadequate for the isolation of slowly growing agents with long incubation periods. The same would certainly be true of most work on other subacute and chronic nervous system degenerations, such as multiple sclerosis, amyotrophic lateral sclerosis, subacute sclerosing leucoencephalitis, subacute inclusion encephalitis, etc. It was with this in mind that in 1959 I decided to resume our search for a transmissible agent in kuru, and to enlarge the program at the same time toward a similar attack on a great many other human diseases which should be similarly reinvestigated.

The plan was for inoculation of unimpeachably adequate inocula, i.e., human brain biopsy material or very early autopsy specimens containing viable cells, inoculated without delay, or, if not so promptly inoculated, frozen promptly to -70° C. (dry ice) or in liquid nitrogen and inoculated at a later, more convenient time. The program was planned to include inoculation of many species of primates, including the chimpanzee, and long-term observation of these primates for, at least, 5 years after inoculation. The inocula obtained would also be studied in a wide variety of tissue cultures, small laboratory animals, embryonated chick eggs, in all cases utilizing long-interval blind passage and indirect methods for detecting virus growth, such as fluorescent antibody staining, hemadsorption, competitive viral inhibition, and the production of an antigen detected by complement fixation or hemagglutination inhibition, etc. Attempts to maintain and cultivate viable cells in tissue culture explants from the inocula would be made. Other human tissues including urine, blood, and cerebrospinal fluid, as well as throat swabs and fecal swabs

should be similarly inoculated as facilities permitted, since slow or latent viral agents could well be expected to be present silently in body tissues other than the central nervous system, although their manifestation under certain circumstances was only that of CNS pathology. It was thus that our current program of investigation of slow, latent, and temperate virus infections had its inception in kuru investigations.

The need for a similar approach, admittedly with little reason to expect other than negative results, in the discouraging study of other neurological degenerative disorders of man of unknown etiology was evident: motor neuron disease, in particular the amyotrophic lateral sclerosis associated with parkinsonism-dementia as observed in the Chamorro people in the Mariana Islands, (8); multiple sclerosis, neuromyelitis optica, and disseminated encephalomyelitis, Schilder's disease, and other forms of diffuse cerebral sclerosis; the leucodystrophies, including metachromatic leucoencephalopathy and spongiform leucoencephalopathy; the subacute encephalitides including van Bogaert's sclerosing leucoencephalitis, Dawson's inclusion-body encephalitis, and Pette-Döring panencephalomyelitis; subacute necrotizing encephalopathy; progressive degeneration of cerebral gray matter (Alpers); Jacob-Creutzfeldt syndrome and the presenile dementias (Alzheimer's disease and Pick's disease); myoclonus epilepsy (Unverricht); the hereditary spinocerebellar degenerations; tuberous sclerosis; syringomyelia; essential and post-encephalitic Parkinsonism; torsion dystonia and Halleworden-Spatz syndrome; myasthenia gravis; peroneal muscular atrophy; sympathetic ophthalmia; and also two disorders restricted to the Soviet Union: Kozhevnikov's epilepsy (9) and Vil'yusik encephalitis (10).

There were immediately very practical problems associated with the inception of such a research program. I dislike working in large, highly organized and coordinated scientific programs, particularly those of long duration. Trained as a virologist of acute infections, I found the 5-year incubation period often seen in scrapie in sheep appalling, and even the reduced 1-year incubation period in goats after repeated goat passage of the scrapie virus was of little encouragement. However, what militated most against starting such a project was my own lack of conviction that a transmissible agent would ever be isolated from the disease in question where in all cases complex autoimmune mechanisms, genetic determination, metabolic derangements and extrinsic toxins or nutritional deficiencies had not been excluded as etiologic possibilities. In many cases, particularly with multiple sclerosis, these

appeared more likely possibilities than the infectious hypothesis. Thus, this time-consuming quest for transmissible agents appeared undeniably necessary in all these diseases, but it originally seemed a somewhat unlikely approach which one would prefer to "sell" to more patient and less ambitious colleagues and collaborators and to await their first success with scepticism before entering the field oneself. I had, furthermore, other misgivings: the prolonged association with long-drawn-out, largely negative, observations of inoculated animals, and the discouraging but laborious "failures" represented by the study of each spontaneous disease and infection of our experimental hosts, even the elucidation of senile change in these animals, appeared to me to bode ill for sustained intellectual acuity and virological astuteness in myself and my colleagues, should we launch into such a project. Therefore, in order that we not stagnate in an apathy bred of long incubation periods and negative results, I sought from our veterinarian colleagues, whose progress in this field had already established a sizable array of transmissible slow viral infections, a few of their viruses to serve as appropriate laboratory models for our work with the hypothetical viruses causing human disease. Intellectually stimulating work with "real viruses" would protect us from ennui and provide a promising prospect for the discoveries and publications which are so needed in the gamesmanship of grantmanship, or at least as status symbols by present day investigators.

Björn Sigurdsson first defined the concept of slow infections as a progressive pathological process caused by an agent that remained clinically silent for a long incubation period of months to years, and then produced disease with chronic accumulating disability (11). This was a useful definition to focus attention at the long persistence of the virus in the body as opposed to the pattern we had learned to expect in acute, self-limited viral infections in which the virus disappears from the body after full immune response is achieved. It furthermore phrased the distinction between latency, masking, or the silent carrier state and the slowly progressive destructive effect of a virus infection not promptly terminated by the immune mechanism. It was, thus, no accident that most of the slow virus infections first defined were infections of sheep in Iceland where Sigurdsson worked. This group of animal infections now includes scrapie and its Icelandic variant, rida (11); visna (7) and maedi (11); infectious adenomatosis of sheep (12); and Aleutian mink disease (13). Of these, scrapie was the first to be established as a transmissible phenomenon (14). The apparent genetic dependence of suscep-

tibility to scrapie in sheep and the remarkable heat stability of the agent suggested unusual properties of the virus. Chandler's discovery that a scrapie-like disease could be transmitted by inoculation of scrapie goat brain into laboratory mice offered a promising laboratory model, using a known virus (15), which would serve to make extensive experimental studies possible, thus keeping investigators in this field alert and presumably leading to discoveries applicable to our study of the human disease. I, therefore, visited the principal laboratories working with scrapie, the Compton and Moredun and Wellcome laboratories in the British Isles, and obtained from Mr. Pattison at Compton the ninth passage scrapie in goat brain to initiate our work in mice. Dr. Morris and I subsequently isolated in mice a strain of scrapie from the natural disease in American sheep (16). From Dr. Hotchin we obtained our second laboratory model in his persistent tolerant infection of mice with lymphocytic choriomeningitis virus (17).

In later papers of this workshop-symposium, Dr. Gibbs and I shall review the current status of our search for transmissible agents in human diseases listed above (18) and of our studies on the virology of the scrapie agent (19, 20); and Mrs. Beck will report further new findings in scrapie neuropathology in the mice we have sent to her, and still further analogies between scrapie and kuru—the intriguing analogy which provided the inception for this program and for the current symposium (21, 22).

Parenthetically, I should account for the naming of our section and of this workshop: the words "slow" and "latent" in the title need no defense in dealing with diseases of long incubation periods, but, the use of the word "temperate" is—or I fortunately can now say, was—less easily justified at the time we chose it for its restrictive meaning in virology. The term was then appropriately applied to only those bacterial viruses which existed as integrated provirus in the cell, only to be "induced" or activated to a vegetative state of reproduction by external chemical or physical factors. The term had been used by bacteriophage workers only for viruses which could occupy loci as a genome on the bacterial chromosomes, as their work with the transduction phenomenon had demonstrated. Whether any mammalian viruses fulfilled these requirements for temperateness remained yet to be established. However, our choice of the term has proved, I believe, not to have been very premature, as recent work of the cancer virologists, who also work with slow, latent, and temperate viruses, has demonstrated.

It behooves us to review classical virology before launching into the study of "natural" slow, latent, and temperate viruses, from the point of view of slow, subacute, chronic, latent, masked, recurrent, and long incubation infections and of the carrier state—all well known phenomena already studied at the dawn of virology. These are neither new ideas nor new problems, nor do they necessarily involve "new" viruses.

Viruses Producing Slow, Latent, or Temperate Infections

The problem of the long survival or persistence—latency, or masking, if one wishes—of viruses in their mammalian hosts may be given wide biological perspective from a consideration of non-mammalian cells harboring viruses. We should not lose sight of the fact that most plant virus infections are slow or chronic, that many are inapparent infections, and that, as in the case of tobacco plants infected with tobacco mosaic virus, a considerable portion of the intracellular protein may chronically be the virus protein.

Streissle and Maramorosch's demonstration of antigenic similarity between the wound tumor virus infecting plants and transmitted by an agallian leafhopper vector, and the reoviruses of mammals to which the wound tumor virus is similar in electromicrographs, the possession of double-stranded DNA, and other physical properties, should give us ample scope for wide flights of fancy (23). The transovarian transmission of virus in ticks and leafhoppers from one generation to the next must certainly also have bearing on the problem of viral survival in vivo (24). Pamela Abel's report of the propagation of vaccinia virus in *Bacillus subtilis* (25), Bayreuther and Romig's successful cultivation of polyoma virus in the same bacterium (26), and Sander's production of bacteriophage in tobacco leaves (27) forces us to span the entire spectrum of all viruses and all hosts from man to lower plants in our thinking about the phenomenon of latency. The long survival of mammalian viruses in their arthropod vectors and the silent infection of these vectors, of snakes and of birds with arboviruses; the silent infection and long persistent carrier states of bats with rabies, and of rodents with many of their latent viruses, including tumor viruses; and the isolation of latent viruses from the tissue cultures of monkey kidney cells used in poliomyelitis vaccine production, amply reflect the scope of the problem of slow, latent, and temperate virus infections in mammals.

I do not here propose to develop this theme any further than to list those mammalian viruses which

we all know to fall into these categories (table I). It is interesting to note how few viruses have been isolated over 2 months after initial contact with the patient in the case of man—if we raise the period to 6 months or 1 year it would become still fewer.

Isolations of Viruses From Chronic Central Nervous System Diseases of Man

In spite of numerous reports and suggestions of virus etiology the verified and reliable demonstrations of viruses in the central nervous system in subacute or chronic human diseases have been very rare. Soviet workers have reported the isolation from brain biopsy of the Russian spring-summer encephalitis virus in a patient with Kozhevnikov's epilepsy. To my knowledge such an isolation has not been repeated in the Soviet Union. Russian spring-summer encephalitis antibody is found rather regularly in Kozhevnikov's epilepsy patients, who often give a history of antecedent acute encephalitis (28). The earlier reputed virus isolation from patients with multiple sclerosis in the Soviet Union (29) was shown subsequently to be a strain of rabies virus (30). Soviet workers have more recently reported isolation of virus from Vilyuisk encephalitis in Yakutsk people in Siberia (31). The virus in Casals' hands appears to be a member of the mengo group of mouse viruses and its origin from the human material is thus unverified (32). Failure to find antibodies to the isolated viruses in more than a few of the patients increases suspicion that they may not be of etiological significance. Zil'ber in Moscow has reported a motor neuron disease in monkeys, appearing 3 years after inoculation of the macaques with human brain tissue from amyotrophic lateral sclerosis victims, and serial transmission of the disease in two passages with long incubation periods (33). Several authors have recently reported scrapie-like disease in sheep after their inoculation with brain material from a patient with subacute encephalitis (34), and from patients with multiple sclerosis (35).

There are reports of herpes simplex virus isolation from subacute encephalitis and meningoencephalitis, although the convincing isolations have all been from rather acute diseases, rather than from any lingering clinical syndromes (36). Cytomegalic inclusion virus has been demonstrated in several infants who were chronically ill for a long period (37). Isolations in Europe of viruses of the encephalomyocarditis group from subacute cases of the Guillain-Barré syndrome have not proved the etiological role of these agents, nor

TABLE I.—List of Mammalian and Avian Viruses Demonstrating Slow, Latent, or Temperate Behavior

Virus (with host or other restriction)	Prolonged inapparent infection or "carrier-state" (no symptoms or gross pathology)	Progressive slowly spreading disease: (progressive pathology) ¹	Long incubation period (over 2 months) ²	Recurrent acute infections (beyond period of primary immune response) ³	Virus (with host or other restriction)	Prolonged inapparent infection or "carrier-state" (no symptoms or gross pathology)	Progressive slowly spreading disease: (progressive pathology) ¹	Long incubation period (over 2 months) ²	Recurrent acute infections (beyond period of primary immune response) ³
IN MAN					IN ANIMALS				
Rabies			+		Herpesviruses:				
Homologous serum jaundice	+		+		H. simiae (B virus)	+			
Infectious hepatitis	+		+	+	H. tamarinus (marmosets)	+			
Herpesviruses:					Infectious bovine rhinotracheitis (pustular vulvovaginitis)	+			+
H. simplex	+	+		+	Infectious equine rhinotracheitis	+			+
H. zoster (? reactivation of chickenpox virus after drugs or trauma)	+			+	Equine rhinopneumonitis (equine abortion)	+			+
Herpes-like virus from Burkitt's lymphoma		+			H. caniculi (virus III)	+			
Cytomegaloviruses (cytomegalic inclusion disease; salivary gland virus)		+			H. suis (pseudorabies)	+			
Basophilic viruses:					Canine herpes	+			
Psittacosis-lymphogranuloma group:					Feline rhinotracheitis	+			
Lymphogranuloma venereum		+			Salivary viruses of:				
Psittacosis	+				Guinea pigs	+			
Cat scratch disease		+			Rats	+			
Trachoma group:					Mice	+			
Trachoma		+			Chinese hamsters	+			
Inclusion conjunctivitis (inclusion blennorrhoea in adults)		+			Australian opossum	+			
Poxviruses: Molluscum contagiosum		+			Chimpanzee	+			
Rubella (in newborn)	+				Laryngotracheitis of chickens	+			
Adenoviruses (types 1, 2, 5, in adults and newborn)	+				Herpes of:				
Reovirus 3 (from Burkitt's lymphoma)		+			Parrots	+			
Arboviruses:					Pigeons	+			
Russian spring-summer encephalitis (Kozhevnikov epilepsy)		+			Owls	+			
Vilyuisk		+			Cormorants	+			
Papovaviruses: warts-verrucae		+			Basophilic Viruses:				
					Psittacosis-lymphogranuloma group:				
					Psittacosis	+			+
					Ornithosis	+			
					Bovine encephalomyelitis	+			
					Enzootic abortion of ewes	+			
					Meningopneumonitis	+			
					Mouse pneumonitis	+			
					Bovine pneumonitis	+			
					Goat pneumonitis	+			
					Feline pneumonitis	+			
					Opposum virus	+			
IN ANIMALS									
Rabies:									
In dogs	+		+						
In bats	+								

See footnotes at end of table.

TABLE I.—List of Mammalian and Avian Viruses Demonstrating Slow, Latent, or Temperate Behavior—Con.

Virus (with host or other restriction)	Prolonged inapparent infection or "carrier-state" (no symptoms or gross pathology)	Progressive slowly spreading disease: (progressive pathology) ¹	Long incubation period (over 2 months) ²	Recurrent acute infections (beyond period of primary immune response) ³	Virus (with host or other restriction)	Prolonged inapparent infection or "carrier-state" (no symptoms or gross pathology)	Progressive slowly spreading disease: (progressive pathology) ¹	Long incubation period (over 2 months) ²	Recurrent acute infections (beyond period of primary immune response) ³
IN ANIMALS					IN ANIMALS				
Poxviruses:					Other mouse viruses:				
Ectromelia.....	+				Theilers GDVII, FA, TO...	+			
Myxoma.....	+				Reovirus 3.....	+			
Fibroma.....	+				K virus.....	+			
Fowlpox.....	+				Lymphocytic choriomenin-				
Rabbitpox.....	+				gitis.....	+			
Monkeypox.....	+				Mouse adenovirus.....	+			
Vaccinia (cattle).....	+				Encephalomyocarditis.....	+			
Myxoviruses:					Mengo.....	+			
Newcastle disease.....	+				MM virus.....	+			
Sendai.....	+				Hepatitis: MHV, JHM.....	+			
SV5—simian hemadsorption..	+				Kraft (diarrhea).....	+			
Distemper.....	+				Vilyuisk.....	+			
Rinderpest.....	+				Arboviruses:				
Tumor viruses:					Western equine encephalitis				
Canine oral papillomatosis..	+				in reptiles.....	+			
Canine venereal sarcoma....	+				Eastern equine encephalitis				
Mouse:					in reptiles.....	+			
Mammary carcinoma					Japanese B encephalitis in				
(Bittner).....	+				birds.....	+			
Mouse leukemias—Gross,					Rio Bravo (bat salivary				
Moloney, Friend,					gland).....	+			
Rauscher.....	+				Powassan.....	+			
Riley virus, LDV.....	+				Machupo (Bolivian hemor-				
Mouse papular agent....	+				rhagic fever virus in ham-				
Polyoma:					sters).....	+			
In mice, rats, hamsters..	+				Bat leucoencephalitis.....	+	+		
Papovaviruses:					Slow virus group:				
SV20.....	+				Visna.....		+	+	
SV40.....	+				Maedi.....		+	+	
Papilloma (Shope).....	+				Scrapie (rida).....		+	+	
Rabbit oral papilloma..	+				Infectious adenomatosis of				
Avian tumor viruses:					sheep.....		+	+	
Rous sarcoma; RIF.....	+				Aleutian mink disease.....		+	+	
Fowl leukemia.....	+				Mink encephalomyelitis.....		+	+	
Myeloblastosis.....	+				Other viruses: Equine infectious				
Erythroblastosis.....	+				anemia.....	+			
Squirrel fibroma.....	+								
Deer fibroma.....	+								
Yaba virus.....	+								

¹ Of course many otherwise acute self-limited infections may become slow and chronic in the face of a breakdown in the immune mechanism as in the case of agammaglobulinemia (e.g., progressive vaccination reaction).

² Arbitrarily selected to exclude the group of viruses causing primary acute infectious disease which have the "long" in-

cubation period of 3–4 weeks (chickenpox, mumps, epidemic hemorrhagic fevers, etc.)

³ To exclude the one recurrence phenomenon seen in many infections with double hump temperature and symptom curves, such as poliomyelitis where the second phase occurs before the complete immune response has taken place.

have they been reported for subjects who have been ill for two months or more (38).

Indirect suggestions of possible virus involvement have been made from immunological observations. Thus, Sabin has demonstrated by immunofluorescence in formalin fixed brain sections what appears to be specific viral fluorescence for herpes simplex virus in several multiple sclerosis patients (39). Adams and Imagawa have shown a higher incidence of measles antibodies in sera from multiple sclerosis patients than in serum collections from control subjects (40). Reed et al. (41), Sibley and Foley (42), and Pette and Kuwert (43) have confirmed this, but Ross et al., while failing to find significantly higher measles antibody incidence in multiple sclerosis patients than in control subjects, have found a higher incidence of antibodies to herpes zoster (44). Thormar has shown antibodies to visna virus in both normal and pathological human sera (45).

This, to my knowledge, is the sum total of virologically acceptable evidence for chronic or subacute central nervous system syndromes attributable to virus etiology to date. In animals the matter is quite different: in scrapie (and rida), visna, Aleutian mink disease, and now in the encephalomyelitis of mink, we have examples of chronic viral infections involving the central nervous system.

Possibility of Virus Participation in Chronic, Exacerbating and Remitting, and Subacute Pathological Processes

There is nothing new in considering virus infection as an etiologic possibility in a large number of chronic diseases of man. However, the development of medical virology in the first half of this century was associated with a vast success in the isolation and characterization of viral agents responsible for acute febrile diseases, whereas in chronic, exacerbating and remitting and subacute processes the virologist was notably unsuccessful in isolating viruses with a few noteworthy exceptions, such as, particularly, the virus of herpes simplex. Thus, in the fifties, those trained specifically in virology were generally more skeptical of viral etiological possibilities in chronic disease than were many clinicians who lacked the full appreciation of how shatteringly negative had been all early attempts to isolate viral agents from chronic progressive degenerative disorders, neoplasms, and other diseases of man. In the current decade the concept that a cell-separable replicating 'package', containing its own genetic blueprint in ribonucleic acid or desoxyribonucleic acid

molecules may cause directly, or be implicated in (perhaps through a complex chain of pathogenic events) a wide variety of chronic disorders has become accepted. It may, therefore, be well to summarize the types of chronic diseases to which such thinking has been applied and to list, specifically under each, those viral agents which are already either completely incriminated or under suspicion.

1. Chronic Hypersensitivity or Auto-Immune Diseases (Collagen or Connective Tissue Diseases)

in man:

- Chronic thyroiditis (Hashimoto's disease)
- Disseminated lupus erythematosus
- Periarteritis nodosa
- Rheumatoid arthritis and Still's disease
- Dermatomyositis
- Scleroderma
- Rheumatic fever
- Sydenham's chorea
- Multiple sclerosis
- Allergic encephalomyelitis (neuroparalytic accidents to Pasteur rabies vaccine)
- Postinfectious encephalomyelitis
- Sympathetic ophthalmia

in animals:

- Experimental allergic encephalomyelitis
- Auto-immune hemolytic anemia and thrombocytopenia in dogs (Lewis)
- Hemolytic anemia in NZB mice

VIRUSES

in man:

- Infectious hepatitis
- lupoid hepatitis leading to disseminated lupus erythematosus (LE cells, anti-DNA and AICF antibodies)
- Yellow fever
- (AICF antibodies)
- Measles, rubella, vaccinia
- postinfectious encephalomyelitis
- Infectious mononucleosis
- (heterophile antibody)
- Non-virus example
- Mycoplasma (cold hemagglutinins in primary atypical pneumonia)

in animals:

- Aleutian disease in mink
- collagen disease pathology
- hypergammaglobulinemia
- Lymphocytic choriomeningitis
- deaths prevented by X-ray or N-mustard inhibition of immune response.
- breakthrough of immune response in "late disease" following primary tolerant infection.

2. Tumors and Neoplasms

in man:

- Burkitt lymphoma in Africa and New Guinea
- Leukemia "epidemics"

2. Tumors and Neoplasms

in man—Continued

Acute blast cell leukemia
Hodgkin's disease; lymphosarcoma
Multiple myeloma
Mycosis fungoides
Salivary gland tumors in Southeast Asia
Primary liver carcinoma in tropics
Epidermoid carcinoma from tropical ulcers

VIRUSES

in man:

Molluscum contagiosum
Warts
Lymphogranuloma venereum
Infectious mononucleosis and infectious lymphocytosis (adenovirus type 12)
Lymphocyte proliferation stimulated
Herpes-like virus from Burkitt lymphoma
Reo virus type 3 from Burkitt lymphoma
Yaba virus (laboratory infection)

in animals:

SV40 and SV20
Polyoma
Adenovirus types 3, 7, 12, 18, 21, and 31
Mouse tumor viruses
mouse leukemias (Gross; Friend; Moloney; Rauscher)
lactic dehydrogenase virus (Riley)
mammary carcinoma (Bittner)
mouse papular agent (Kraft and Moore)
mouse lymphoma (Reo virus 3)
Avian tumor viruses
Rous sarcoma; RIF virus
fowl leukemia
myeloblastosis
erythroblastosis
Shope rabbit papilloma
Rabbit oral papilloma
Canine oral papillomatosis
Canine venereal sarcoma
Squirrel fibroma
Deer fibroma
Yaba virus (monkeys)

3. Chronic and Subacute Degenerations of the Central Nervous System

in man:

Kuru
Amyotrophic lateral sclerosis
Amyotrophic lateral sclerosis with parkinsonism-dementia on Guam
Multiple sclerosis
Subacute inclusion (type A) encephalomyelitis (Dawson)
Subacute sclerosing leucoencephalitis (van Bogaert)
Essential parkinsonism
Postencephalitic parkinsonism
Progressive multifocal leucoencephalopathy

in animals:

"ALS agent" into monkeys (Zil'ber)
Kuru in chimpanzees

VIRUSES

in man:

Herpes simplex
in multiple sclerosis (Gudnadóttir)
immunofluorescence in multiple sclerosis (Sabin)
Papovavirus in progressive multifocal leucoencephalopathy
in animals:
Scrapie, rida
Visna, maedi
Aleutian disease in mink
Mink encephalomyelitis
Bat leucoencephalitis (Thomas)
Rat virus (Kilham)
Bovine encephalomyelitis (bovine pustular vulvovaginitis)

4. Exacerbating and Remitting Acute Disease with Latency or Masking

in man:

Herpetetic syndromes (fever blisters, keratitis, meningoencephalitis, etc.)
Shingles following trauma or drugs
Non-viral examples
Brill's disease
malaria

VIRUSES

in man:

Herpes simplex
Herpes zoster (chickenpox reactivation)
Latency in doubt:
cytomegalovirus
trachoma
infectious hepatitis
warts

in animals:

Herpes viruses
Herpes simplex in rabbits (epinephrine provoked keratitis)
infectious bovine rhinotracheitis
Herpes simiae
Psittacosis

5. Geriatrics: Senility

in man:

Kuru (amyloid plaques akin to senile change developing rapidly in young patients)
Presenile dementias: Alzheimer's disease and Pick's disease

in animals:

Lymphocytic choriomeningitis
reduced average life span in persistent tolerant infection
"late disease"
Scrapie (PAS-positive, amyloid plaques)
Kuru in chimpanzees (plaques)

6. Genetic or Familial Determination of Disease Expression

in man:

- Agammaglobulinemia (with increased susceptibility to prolonged or recurrent infection)
- Chediak-Higashi syndrome (with increased susceptibility and severity of response to infection)
- Kuru in New Guinea
- Amyotrophic lateral sclerosis with parkinsonism-dementia: Chamorros on Guam
- Non-viral infections
 - malaria resistance in sickle cell anemia
 - tuberculosis susceptibility in certain ethnic groups
 - tinea imbricata susceptibility in albinos and families
 - rheumatic fever in certain ethnic groups

VIRUSES

in man:

- Poliomyelitis in certain somatotypes
- Live attenuated measles virus (variable reaction in different races)
- Influenza (variable severity in different races)
- Smallpox (decreased mortality in persons with anti-A iso-hemagglutinins)

in animals:

- Scrapie (and rida)
- Aleutian mink disease (Chediak-Higashi syndrome and albinotic dilution of coat color)
- B group arbovirus susceptibility in mice

7. Congenital Anomalies

VIRUSES

in man:

- Rubella in infancy
- Cytomegalovirus
- Non-viral examples
 - syphilis
 - toxoplasmosis

in animals:

- Rat virus
- H-1 (Toolan)
- Blue tongue (attenuated vaccine in ewes)
- Japanese B encephalitis (swine)
- Equine abortion virus
- Rubella in ferrets
- In inoculated chick embryos
 - herpes simplex
 - influenza
 - measles
 - mumps
 - Newcastle disease
 - vaccinia

This historical account thus traces the origin of our slow, latent and temperate virus study program from its roots in the disease kuru in the highland interior of New Guinea. Kuru remains today an unsolved challenging problem, the solution of which cannot fail but to give us many new leads in neurophysiology and

neuropathology, in the study of the whole range of heredofamilial degenerative disorders of the brain, in human genetics of isolated groups, and even in the pathogenesis of senile changes.

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Amyotrophic Lateral Sclerosis: A Reappraisal

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INTRODUCTION

No single concept of the cause of amyotrophic lateral sclerosis (ALS) has been widely accepted, and this in itself suggests that ALS may be more than a single disease entity (1). Series of cases have been reported suggesting that the causative mechanism or agent may have been heredity or abiotrophy (2), trauma (3), heavy metal or organic intoxicants (4, 5), metabolic (6, 7), or nutritional processes (8), or infectious agents (9-11), the latter associated with prior encephalitis. In the past 15 years, numerous papers have been published describing the research associated with the discovery of the high incidence of the disease in several western Pacific islands, but no cause for these foci has been identified (12-23). More recently, a particularly stimulating series of reports suggesting that ALS may be due to a viral agent have come from Zil'ber and Bunina and their associates in the U.S.S.R. (24-26).

The recognition of foci of ALS in a chain of western Pacific islands—the Marianas (Guam, Rota, Saipan, and Tinian), Japan (Mitogawa and environs (27) in the Kii Peninsula), and western New Guinea (in the Kepi region (28) on the south coast)—have initiated a search for some "common denominator." Factors studied have included the ancestry of the population of these isolated areas through blood group and other genetic markers (29), chemical studies of soil and water (30, 31), a description of exotic plantlife and nutrition (32, 33), and even exposure to migratory birds passing along flyways of these islands since some avian-borne infectious agent could conceivably serve in the focal distribution of the disease (34).

The possibility of an infectious basis for the disease on Guam was considered in 1953, but there was no difference in the results of complement fixation between patients and controls for those viruses which

could be tested, and it was not feasible to attempt the isolation of an infectious agent at that time. The recognition that the parkinsonism-dementia complex (PD), another prevalent disease in the Marianas, was possibly a clinical variant of the local form of ALS and that in both disorders neurofibrillary changes and intracytoplasmic inclusion (granulovacuolar) bodies could be demonstrated (17, 18) resulted in attempts, a few years later, by Dr. Leon Rosen of the National Institutes of Health, to isolate an infectious agent from the brain and spinal cord of recently deceased patients. Tissue from seven patients with ALS or PD were inoculated into suckling and adult small animals and into various tissue cultures. The studies were discontinued when the animals remained well and no growth was present in the cultures after 3 months.

The concept of a possible "slow virus" infection in ALS with a prolonged incubation period was renewed with Zil'ber's work and emphasizes the need for additional thorough search for an infectious agent.

In an effort to identify major specific disease entities which may constitute the syndrome of ALS, the clinical and pathologic features which are common to the various forms will be described in this report and that of Dr. Hirano to follow. We shall then emphasize those variations of a clinical, pathologic, epidemiologic, or genetic nature which suggest that grouping into (1) classic or sporadic, (2) familial and presumably hereditary, and (3) Mariana Islands forms is appropriate (see table I). In addition, I shall review some of the recent studies concerned with the infectious hypotheses of ALS and will present an evaluation of the ALS patients and experimental animals which I had the privilege to observe as a member of a recent U.S.A. Exchange Delegation to the U.S.S.R.

General Features of ALS

Today the term amyotrophic lateral sclerosis (ALS) is used synonymously with "progressive motor system disease" and includes the clinical components of progressive muscular atrophy and progressive bulbar palsy. ALS occurs in adults and is characterized by lower motor neuron degeneration affecting the spinal or the bulbar motor nuclei or both, usually with involvement of the Betz cells of the cortex and demyelination of corticospinal and corticobulbar tracts. This neuronal degeneration results in skeletal muscular wasting and weakness and usually fasciculation, which may affect all skeletal and the lower bulbar levels before respiration or swallowing becomes so impaired that death intervenes. The disease is progressive, and the duration averages about 3 to 4 years; occasionally it may be as brief as 1 year or as long as 10 years or more. Upper motor neuron disease in the form of spasticity and increased or pathologic reflexes is likely to be present during the course of the illness, even in the presence of muscular wasting. Whether upper motor neuron disease is recognized clinically or not, demyelination of the corticospinal tracts with degeneration of Betz cells is usually found at autopsy. Systems other than motor (for example, cerebellar, sensory) are rarely affected, but when they are, they may provide points of distinction in the classification of ALS as used here.

The concept that ALS was a single disease entity and that heredity played a prominent role did not seem unreasonable at the time of the last Mayo Clinic Symposium on ALS (35). At that time, the distinguishing clinical and pathologic features to be described had not yet been recognized by this investigator.

Classic or Sporadic Form

About 90 to 95 percent of the cases observed in the United States occur sporadically and have the features of the classic form of the disease. The site of initial wasting and weakness varies, but in most series is about 40 percent in the upper extremities, about 25 percent each in the bulbar levels and the lower extremities, and about 10 percent in mixed sites (36). This form of disease rarely begins with symptoms of upper motor neuron disease alone. There is no consistent indication of sensory or cerebellar dysfunction, and only rarely has an extrapyramidal or intellectual component been described. The illness begins at any time in the adult years, but rarely before 25 years of age. The mean age of onset was 52 years in the series collected

by Friedman and Freedman (36) and 53 years in the series of Mulder (21). The preponderance of males, about 1.6 to 1, is unexplained. On the basis of available mortality statistics (in which most deaths are due to the classic form of ALS), the rates for the United States by region and for Canada, Australia, and several western European countries do not show any appreciable variation.

The possibility that sporadic cases of ALS reflect some biochemical defect which might be genetically determined was considered in an earlier paper (22). Dermal changes which include increased mucopolysaccharide, connective-tissue disorientation, elastosis, and disorganization of collagen structure were believed to reflect such a biochemical defect. They were noted in more than 50 percent of a sample of sporadic cases in the United States and in the same proportion of cases on Guam (37).

It was suggested in an earlier paper that positive family histories were infrequently reported because of inadequate taking or recording of the history or because the "penetrance" in ALS was ordinarily of a low order of magnitude.

As of the present writing, the cause of the classic form of ALS remains unknown. Although there are reasons to increase efforts to isolate an infectious agent, an equally valid etiologic concept, in my opinion, remains that of exposure to some unidentified nutritional substance which in genetically predisposed individuals behaves as a specific toxin.

Hereditary ALS

Charcot considered the disease as nonfamilial, and a number of series were subsequently reported in which no familial cases could be identified (38, 39). On the other hand, one of the eight patients described in the first report on progressive muscular atrophy, by Aran (40) in 1850, had an extensive family history. Gowers (41) in England and Hammond (42) in the United States had also observed familial cases and considered "abiotrophy," or premature constitutional degeneration of the motor cells, as a reasonable explanation for the disease. Furthermore, at least 20 families with extensive histories of ALS were reported (2) before 1950.

Before the studies on Guam, ALS was considered by almost all neurologists as nonfamilial and nonhereditary. Because of the familial aggregations of ALS observed on Guam, new approaches to the question of heredity in the disease developed. Two series, totaling 159 patients, were collected at the Mayo

Clinic, and among these a positive family history was noted in about 7 percent. When several of these families were thoroughly studied, a pattern of dominant inheritance with high penetrance was noted. In one of these studies on the possible role of genetics in ALS, it was noted that, in sibships with at least 1 affected member, about 50 percent of the siblings (88 of 179) had experienced the disorder (2). In the last 10 years, many additional family histories have been reported from several countries in Europe, from the United States, and from Japan (43-49). Haberlandt (43) has made a particularly thorough evaluation of familial aggregation in his cases and indicated that perhaps as many as 12 percent of the ALS patients who came to his attention had a family history of the disease.

In almost all kindred reported, family incidence is not limited to a brief period of time when various members might be exposed to a common agent, but is spread over generations. In some cases, the disease

continued to occur in families in which some members had migrated from Europe to America.

The pedigrees which have been reported are impressive and might have led to the conclusion that all cases of ALS were genetically determined. However, this could be misleading, for there is a tendency among physicians to report or recall not the usual kindred but the exceptional ones. An analysis of the pedigrees, however, reveals that the range of ages of onset of ALS did not differ from those of the sporadic cases in the United States or in the cases on Guam. Mean age of onset is slightly less than that of sporadic cases, but in the examination of the individual patient, this information would be of no particular differential value (table I).

In the individual familial cases of ALS, nothing distinctive can be detected in clinical examination. However, I have the impression that the familial cases are more likely to begin with weakness and wasting in the lower extremities than is generally observed in the

TABLE I.—Selected Clinical and Pathologic Features of Amyotrophic Lateral Sclerosis

	Sporadic cases	Familial cases	Mariana cases
Approximate mean age at onset, year (Usual range)	52 (25-65)	47 (25-65)	45 (25-65)
Sex ratio, M:F	1.6:1	1:1	2:1 ¹
Approximate mean duration, year (Usual range) ²	3 (1-8)	3 (1-10)	3 (1-8)
Clinical features:			
Initial symptoms lower extremities, percent	20-25	40-50 ³	20-25
Initial symptoms of spasticity as in primary lateral sclerosis	Rare	Rare	About 10 percent
Extrapyramidal symptoms	Rare	Occasional	Frequent (if cases with both ALS and PD are included)
Dementia present	Rare	Occasional	Infrequent in ALS alone; frequent in ALS-PD
Sensory changes	Not detected	Not detected	Not detected
Histochemical changes in skin (collagen)	More than 50 percent	Infrequent(?)	More than 50 percent
Pathology in central nervous system:			
Posterior column demyelination and column of Clarke changes	Rare	About 50 percent	Rare
Neurofibrillary changes before age 60 years	Rare	Rare	Common
Granulovacuolar bodies	Rare	Rare	Occasional

¹ Patients with PD have a mean age of about 53 years and a sex ratio of about 2.5:1, M:F.

² Since 1950, the reported mean duration has increased about 1 year in the Mariana cases to 4 years; this may apply to

other groups as well and may be related to improved supportive therapy or the earlier recognition and more accurate establishment of the date of onset.

³ Clinical impression of the author.

sporadic cases. In contrast to the observed preponderance of males in the classic form, the familial cases have shown a 1:1 sex ratio (2). The histochemical changes observed in the skin of the sporadic and Mariana Islands patients as described above have not yet been noted in a few patients with familial ALS who have been studied.

Physical examination does not disclose any sensory changes in the familial cases. Pathologic study has also shown nothing distinctive in about half the cases that have come to my attention. However, in the other half, and particularly in those with initial symptoms in the lower extremities, posterior column demyelination in the midroot zones and cell loss in the column of Clarke was found at autopsy. The changes in the posterior column resemble those seen in the hereditary ataxias and suggest that, in the "spectrum" of neurologic system diseases, some of the familial cases may shade into the ataxia group. Some of the unusual histologic features observed in familial cases will be described by Dr. Hirano.

Mariana Islands Form of ALS

The early studies on Guam and the other Mariana Islands revealed that about 10 percent of the deaths among the adults in the indigenous population (Chamorros) were due to a form of ALS which was identical in its clinical and pathologic features with the classic form of the disease (14). Among the Chamorros, the disease had been considered as familial for decades and perhaps for more than a century. Because of the stigma associated with the disease in this area, detailed family histories were often difficult to obtain. Furthermore, illegitimacy was frequent, and most of the earlier medical, church, and government records had been destroyed during World War II. No clear pattern of inheritance could be identified; it was uncertain whether this was because of the practical reasons mentioned above or because the Mariana Islands form of the disease, although often familial, was not hereditary (34).

At the termination of the earlier series of investigations on Guam in 1956, no exogenous factor had been identified. It was tentatively concluded that ALS, at least among the Chamorros, was inherited, perhaps as an autosomal dominant trait, because of the following reasons: ALS was exceedingly prevalent in the Chamorros whether they lived on Guam or the other Mariana Islands, or had migrated to California (50). With the exception of one or two cases, the disease was not known in the other ethnic groups on the same islands. Although the pedigrees could

not be analyzed satisfactorily, familial aggregations, often over several generations, were known (13). ALS involved one "system" of the central nervous system as some recognized hereditary disorders are known to do; also, numerous pedigrees of ALS in which the disease behaved as an autosomal dominant trait had been reported.

Since 1957, there have been several developments which disclosed previously unrecognized features of the disease and led to a renewed search for an exogenous factor. These developments were the following:

1. The recognition that another and ordinarily rare neurologic picture referred to as parkinsonism-dementia complex (PD) is highly prevalent among the Chamorros (20). Pathologically, in PD, neurofibrillary changes and granulovacuolar bodies are common (as Dr. Hirano will report); this same type of unusual neuronal change was also belatedly recognized in the Mariana Islands cases of ALS (17-19).

2. The observations that among the relatively small non-Chamorro population on Saipan and Tinian, and among the few long-term non-Chamorro residents on Guam, ALS was occurring more often than it should if the disease were unduly prevalent only among the Chamorros (51). In one non-Chamorro case studied at autopsy, the neurofibrillary changes which had previously been noted among the Chamorros were also present (52).

3. A new focus of ALS was reported in another area of the western Pacific, namely, the Kii Peninsula of Japan (53). One of these patients at autopsy also had the unusual pathologic changes observed in the "Marianas" cases (54).

4. In one other possible focus, in Kepi, a small remote village near the southern coast of western New Guinea, Gajdusek (28) noted the frequent occurrence of muscular atrophy in the hands of adolescents and young adults and described several cases of motor neuron disease. (This disorder is possibly ALS, but pathologic confirmation is required.)

5. The observation that diaphysial aclasis (multiple exostosis) and also hyperuricemia and perhaps hyperglycemia were also prevalent in the Chamorros and Carolinians of the Marianas (55, 56). Although the association of these disorders with the neurologic disease is uncertain, the possibility of a common toxic factor must be considered.

In table I, the characteristics which now appear to distinguish the "Marianas" form of ALS from the others are outlined. Although the range of ages is the same, the mean age of onset of ALS in the islands is

somewhat less than that of the sporadic and perhaps of the familial cases. The sex ratio reveals a preponderance of about 2 males to 1 female. The mean duration of the disease does not appear to vary by type, nor is there a difference in the major pathologic features of anterior horn cell degeneration and demyelination of the long motor tracts. However, neurofibrillary changes and granulovacuolar bodies are common in the Marianas form of the disease but are observed infrequently in other cases. The histochemical changes observed in the skin of sporadic cases have also been noted in the cases in the Marianas but not, to date, in the few familial cases studied. Clinically, about 10 percent of the Guam cases have spasticity even before muscle wasting becomes apparent (57), whereas this is rare in the other forms. About 10 percent of the Marianas ALS patients have evidence terminally of either extrapyramidal or intellectual deficits. This association of motor neuron, extrapyramidal, and dementia features which is not uncommon in the Marianas cases has been known to occur in both the sporadic and the familial cases of ALS. There are several such reports from Europe and the United States in which parkinsonism or dementia, or both, are present in patients with ALS (58-65).

In view of the fact that the disease is no longer believed to be limited to the Chamorros of these western Pacific islands and since it is highly unlikely that the foci in Japan, the Marianas, and New Guinea are due to a single genetic identity of these peoples, there has been a renewal of efforts to find some exogenous factor common to these areas. The combination of prevalent neurologic disease, the collagen changes in the skin of such patients, and the multiple exostosis which has been observed on Guam led to intensive efforts to find some local food with lathrogenic properties. None has yet been identified. However, one source of toxin which has aroused considerable interest has been the nut of *Cycas circinalis* (Cycad), which apparently has been an important source of starch on the island for centuries. In its natural form, Cycad contains a remarkable water-soluble hepatotoxic and carcinogenic agent, the aglycone of cycasin, which can be removed by thorough elution. Several species of animals have been tested with the aglycone, which chemically resembles dimethylnitrosoamine (32). In large enough quantities, the aglycone is responsible for hepatic necrosis and rapid death; in smaller quantities, it will produce cirrhosis; and in minute quantities, it has produced hepatic and other tumors after a latent period of a year or more (33). It is conceivable that, in the Marianas patients, minute quantities of such an

agent, retained because of inadequate washing procedures of the nut, could lead to some hepatic disturbance in the production of an enzyme which is essential for the metabolism of the motor neurons. Although none of several species of small experimental animals developed central nervous system disease, it has been noted recently that cattle in Australia and Santo Domingo fed on Cycad plants developed neurologic dysfunction. Demyelination of the posterior and lateral columns was present, particularly in the spinal cords of the Australian cattle, according to Innes (66). The possible roles of other nutritional agents, of heavy metals such as manganese, and of an infectious mechanism, are still being explored.

Comments on Hypotheses of the Infectious Nature of ALS and Personal Observations in the U.S.S.R.

Until the report of Zil'ber and associates (26) on the transmission of ALS to rhesus monkeys, evidence that the disease is of viral origin was tenuous. Acute anterior poliomyelitis has been reported as preceding, often by many years, the onset of ALS, but these could be chance associations. Whether such cases reflected predisposition to ALS by the earlier poliomyelitis or were an indication of reactivation of a quiescent infection is uncertain. Zilkha (67), in the symposium on ALS at the Royal Academy of Medicine, in London in December 1962, reported that the proportion of ALS patients he had observed who had had anterior poliomyelitis was greater than one would expect in the population at large. Other neurologists who discussed his report could not support this relationship. The association of prior poliomyelitis was not noted in the Mayo Clinic series of ALS cases (35). In the recent studies on Guam, patients with ALS had the same serum antibody levels to poliomyelitis as did control subjects (68). Furthermore, there was no recognized poliomyelitis on Guam until a serious epidemic occurred in 1899, whereas ALS was apparently first recognized on Guam early in the nineteenth century.

Konovalov and associates (25) described at length a "subacute anterior poliomyelitis" in several adult patients who had been maintained in respirators somewhat beyond the usual termination point of the disease. They referred to these cases as "motoneuraxitis with cytoplasmatic inclusions and retrograde degeneration of the motor pathways." They stated, "This is an autonomous disease due to virus infection." They considered their cases as "dissimilar to all known forms of progressive amyotrophy" because "the course of the

disease is the extension of paresis and atrophy from the affected segments to the ascending adjacent segments * * * or else simultaneously both upwards and downwards extension." Although they considered these cases as distinct from so-called chronic poliomyelitis, the basis for any such differentiation is vague.

In my opinion, neither the chronic poliomyelitis as described several decades ago nor the cases which this group describes as subacute anterior poliomyelitis can be distinguished from progressive muscular atrophy or ALS. This opinion is based on a critical evaluation of the clinical and pathologic description of the patients, as well as our own evaluation of the central nervous system tissues of such a case. The newly described oxyphilic intracytoplasmic inclusion bodies which Bunina (24) and Konovalov (25) noted, first as a feature of hereditary ALS in the U.S.S.R. and later in some of the nerve cells of the patients with "subacute anterior poliomyelitis," have now been observed in sporadic cases of ALS from New York as well as in cases from Guam. (The details will be given in the report by Dr. Hirano that follows.)

Greenfield and Matthews (11) described two cases in which amyotrophy followed the onset of postencephalitic parkinsonism. They also reviewed earlier reports of amyotrophy which had been attributed to epidemic encephalitis. They dismissed many of the cases because they were "localized, radicular, and nonprogressive, and usually evident immediately after the acute attack." "In a further group of cases the clinical picture has not resembled motor neuron disease, the wasting being hemiplegic, remittent, or accompanied by sensory loss." They referred to several case reports "of a clinical syndrome closely resembling motor neuron disease coexisting with undoubted evidence of chronic encephalitis" which they felt were not attributable to a chance association of the two diseases.

The more recent report of Kuimov and Dubov (10) in western Siberia described several cases of ALS in which tick-borne encephalitis was thought to have preceded the onset of muscle wasting and weakness. There was not adequate serologic or virus-isolation confirmation in these cases; also, there was no indication that the geographic distribution of ALS in the Soviet Union is related to that of tick-borne encephalitis (69).

The work by Zil'ber and associates (26) appears to have been stimulated not only by reports such as that of Kuimov and Dubov (10) but also by the work of Bunina (24) and Konovalov and associates (25) on the oxyphilic intracytoplasmic inclusion body in ALS.

In their studies, Zil'ber and his associates inoculated seven monkeys intracerebrally with homogenates of spinal cord and medulla from six patients who had died of ALS. Monkeys inoculated with material from three patients remained unaffected, whereas monkeys inoculated with specimens from the other three patients developed asymmetrical muscular atrophy of the upper and lower extremities with increased tendon reflexes. The disease progressed over a period of 8 months to 3 years in these animals. Homogenates from the brain of affected monkeys produced disease in other monkeys; in one case there were two successful passages after the initial experiment. The morphologic changes in the monkeys were said to be similar to those observed in patients who died of ALS. The changes were found to be less intense in the monkeys, presumably because they were killed before the disease had reached an advanced stage. However, the same inoculum was nonpathogenic for mice and other laboratory animals.

As a member of the recent U.S. Exchange Delegation on Chronic Infections of the Nervous System to the U.S.S.R., I had the opportunity to examine a few patients with ALS in several cities in the Soviet Union. On the basis of these observations and discussions with Soviet neurologists, no apparent difference emerged in the clinical picture or the outcome of the disease as it occurs in the U.S.S.R. and the U.S.A.

At the Gamalaya Institute of Epidemiology and Microbiology in Moscow and at the Institute of Experimental Pathology in Sukhumi, members of the exchange delegation examined several monkeys which had been inoculated some months before with brain homogenates from the monkeys affected earlier (70). The monkeys had asymmetrical atrophy and weakness of the lower extremities; no atrophy or fasciculation of the tongue, other bulbar musculature, or upper extremities was noted. Brisk tendon reflexes were observed in inoculated as well as in uninoculated monkeys. The Soviet workers had reported involvement of the upper extremities but not of the bulbar musculature in monkeys which had been previously inoculated. The delegation noted that spontaneous neurologic disorders were occurring in the monkey colonies in Sukhumi, but the cause and pathologic nature of these illnesses has not yet been systematically studied. In Sukhumi, where the inoculated monkeys were held, they were not isolated from one another or from uninoculated animals during the incubation period of the disease.

Although the members of the delegation observed that inoculated animals manifested signs of motor dis-

ease, they were unable to find unequivocal pathologic changes in the central nervous system of the animals that had received the ALS material. To date, no microscopic studies of muscle or peripheral nerve are available and no electromyographic studies have been reported. Therefore, neither clinical nor pathologic localization of disease in monkeys appears to have been definitely established. Since all monkeys have been inoculated with crude brain homogenates, the physical properties of a "transmissible" agent have not been determined. Serologic and immunologic confirmation is needed, and the research in the U.S.S.R. is continuing to that end.

COMMENT

It has occurred to others as it has to this investigator that ALS is a syndrome rather than a disease entity. The effort to classify ALS into several meaningful groups is of more than academic interest in spite of our inability to influence the outcome of any case at this time. Although the major clinical and pathologic features of the three groups described above appear to be identical, the underlying pathogenic mechanism may be dissimilar if the distinguishing features referred to are real. A possibility exists, of course, that the groupings are artificial and that all these cases fall into place in the spectrum of a disease which has a broader range of variability than is usually accorded it. Furthermore, it is conceivable that the "hereditary" and sporadic forms may each include more than a single disease entity. Perhaps the opportunity afforded on Guam and the other western Pacific islands in the detailed examination of so many patients within such a brief period of time offers us a rare view of the spectrum of such a single disease entity, segments of which are only occasionally recognized elsewhere. If this is the case, is the disease so prevalent in these islands because of an increased exposure to some subtle toxic, infectious, or other exogenous agent which is common there but infrequently encountered in other countries? Or is it due to a common genetic trait of increased predisposition in several apparently different ethnic groups inhabiting this long chain of western Pacific islands? Or is it due to a combination of increased genetic susceptibility and an exogenous factor which is more prevalent here than in other parts of the world?

Even if the Mariana Islands form of ALS is distinct, the solution of the problem there will not only benefit the local population but can be expected to provide insight into the other forms and facilitate their management. Every effort should be made to clarify the

situation on Guam and the other islands where conditions are so well suited for intensive epidemiologic studies. This should include renewed efforts at determining the pattern of disease within the population of the Marianas, including the non-Chamorro residents. Comparisons of environmental conditions in the islands, villages, and population subdivisions which have different incidences of disease are called for.

Further studies under more rigidly controlled conditions are indicated in an effort to determine whether the infectious hypothesis proposed by Zil'ber and his associates in the Soviet Union is correct. A cooperative program has developed between Zil'ber's laboratory in the U.S.S.R. and the laboratories of Drs. Hirano, Gajdusek, and Gibbs at the National Institutes of Health. This has resulted in an exchange of tissues from the affected experimental animals in the U.S.S.R. and from patients on Guam and elsewhere in the U.S.A. Dr. Bunina in Moscow and Dr. Hirano have exchanged tissue blocks for histologic comparisons of human cases from the U.S.S.R. and Guam; Dr. Bunina has also made available to Dr. Hirano specimens from affected monkeys for the independent evaluation that he will report in the paper to follow. International cooperative efforts of this nature offer the promise of a more rapid solution to this illness.

SUMMARY AND CONCLUSIONS

Lower and usually upper motor neuron degeneration is the major feature common to the three groups of amyotrophic lateral sclerosis (ALS) which were described herein as (1) classic or sporadic, (2) familial and presumably hereditary, (3) the Mariana Islands form. Although there is no distinctive clinical feature for a single patient in any of these three groups, there are characteristics which collectively appear to distinguish the three groups.

In the hereditary form (1) there is no predilection for males as in the other two forms; (2) there is a greater likelihood that the initial weakness and wasting will occur in the lower extremities; (3) demyelination in the posterior columns is more likely to be found, although no sensory changes have been detected; (4) the histochemical changes in the skin described in the other forms have not been noted; and (5) a family history is likely to reveal a pattern compatible with dominant inheritance.

In the Mariana Islands form (1) the age of onset of ALS is, on the average, a few years less than that in the classic form; (2) spasticity is more likely to be present initially than in the other forms; (3) extra-

pyramidal features and dementia are more likely to be present terminally; and (4) neurofibrillary changes and granulovacular bodies, which are infrequently noted in neurons of the other forms, are relatively common in these cases.

Although earlier hypotheses supported the concept that ALS on Guam was of a genetic origin, the newly recognized high incidence of the disease in other populations in the western Pacific islands casts some doubt on that hypothesis and demands intensification of efforts to identify a possible exogenous factor. Although recent studies in the Soviet Union support the concept of infection, a toxic nutritional agent which may be superimposed on genetic predisposition still appears to be a reasonable explanation, at least for the focus in the Marianas.

The question of whether ALS is more than one disease was discussed, and the implication of the singular versus multiple disease concept was reviewed. There is a need for further epidemiologic study in the western Pacific islands and for genetic studies of the disease elsewhere. Continuing collaborative programs among scientists in different parts of the world should be facilitated, for they promise a more rapid elucidation of the pathogenesis of this illness.

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DISCUSSION

FIELD: Did you investigate the blood groups in your cases of amyotrophic lateral sclerosis on Guam?

KURLAND: Studies have been carried out both in Guam and in a series here in the States. These will be reported in the near future. There appears to be only an association with the P group. In general, there is nothing dramatic in terms of an association with blood groups.

FIELD: Dr. Vejjajiva in Newcastle did look into 40 absolutely certain cases out of the total of 160 we had in the neighborhood and found a high preponderance of B, statistically significant to the .001 level. This is quite remarkable. As you know, in Guam B is rather high in the population, and this makes the finding even more interesting.

KURLAND: The series of about 300 cases in the Veterans Administration, studied by Myrianthopoulos, did not show any significant difference that I can recall in the ABO system.

GAJDUSEK: There is a problem with these correlations of disease incidence with blood group frequencies in primitive communities, because there is probably no small isolated tribal group where, if you use all the available genetically-determined blood and serum factors, the inbred group will not show an unusually high incidence for at least one. Therefore, a priori, any disease that is high in incidence in such a group will eventually be correlated with whatever blood group or serum factor is in unusually high incidence in that particular isolate merely because of this phenomenon of inbreeding and isolation. Certainly the correlation reflects genetic influences, but it may reflect them so indirectly as to be hardly meaningful.

Pathology of Amyotrophic Lateral Sclerosis

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Since the first description of the pathology of amyotrophic lateral sclerosis (ALS) by Charcot nearly a century ago, numerous hypotheses have been proposed regarding the cause and pathologic mechanism of this illness. Yet the etiology remains obscure.

In the preceding presentation (by Kurland) the extraordinary incidence of ALS among the native population of the island of Guam was described. The concept of subacute or chronic poliomyelitis was reviewed. Also the recent studies on ALS reported in the Union of Soviet Socialist Republics (U.S.S.R.) regarding both human cases and experimental efforts to transmit the disease to monkeys and other species were reviewed, as these suggested a possible viral cause for ALS. In this presentation the following five points will be covered:

1. The histopathologic findings in classic, sporadically occurring cases of ALS based on a review of material at the Montefiore Hospital, New York.

2. Familial ALS in which the classic findings are frequently noted, but in some cases of which posterior-column demyelination and other atypical changes may occur.

3. The histopathologic features observed in cases of ALS on the island of Guam, with emphasis on the frequent presence of Alzheimer's neurofibrillary changes (fig. 1) and granulovacuolar bodies (fig. 2), which are infrequent findings in classic or familial cases.

4. The neuropathologic features of parkinsonism-dementia (P-D) complex on Guam, which demonstrates some striking similarities to the features of ALS

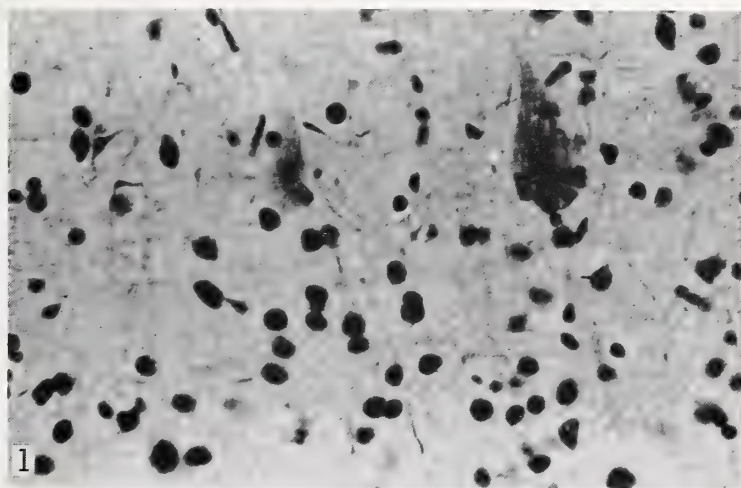


FIGURE 1.—Betz cells with Alzheimer's neurofibrillary changes in a case of amyotrophic lateral sclerosis (ALS) on Guam. (Silver impregnation; $\times 400$.)

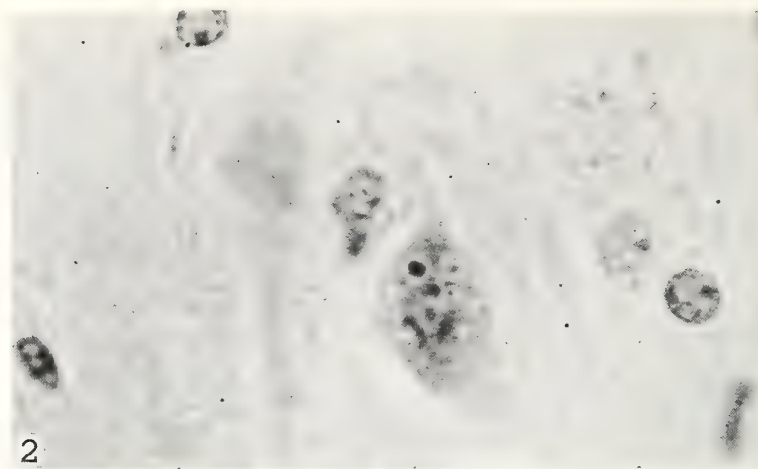


FIGURE 2.—Granulovacuolar bodies in pyramidal cell of Ammon's horn in a case of ALS on Guam. (Hematoxylin and eosin; $\times 700$.)

on Guam. Neurofibrillary changes are present in the substantia nigra, where Lewy's bodies are also occasionally seen. Pyramidal cells in Sommer's sector also reveal appreciable neurofibrillary changes, particularly in the P-D cases, so that electron microscopic examinations of postmortem material were attempted and will be illustrated.

5. A few specimens have been received from Bunina and Zil'ber and their colleagues in the U.S.S.R., who support the concept that ALS is due to a virus. The eosinophilic inclusion bodies originally described by Bunina and associates of Moscow in hereditary ALS and in a few sporadic cases in patients maintained in respirators beyond the usual life expectancy were observed. Similar observations in cases from Guam and New York are presented. The results of our study of spinal cord sections from the experimental monkeys used in studies by Zil'ber and colleagues in Moscow will also be presented.

1. *Sporadic ALS*

The histopathology of ALS has been described in the classic case as degeneration and loss of neurons in the anterior horns of the spinal cord, in the motor nuclei of the brain stem and, in many instances, in the Betz cells of the cortex. Paucity of neurons in these involved areas is associated with astrocytic gliosis. Atrophy of muscles and demyelination of the anterior roots and anterolateral columns of the spinal cord, especially the pyramidal tracts, are constant features. In these demyelinated zones, axons are also affected. Often there is a large number of macrophages which contain abundant sudanophilic lipid granules. Astro-

cytic gliosis is also associated with the tract degeneration.

2. *Familial ALS*

An inherited neurologic disorder, clinically indistinguishable from ALS and occurring in two families, was reported by Engel, Kurland and Klatzo (1) in 1959. Pathologic examination of one male in the first family and a male and female in the second showed severe degeneration of the lower and upper motor neurons. In addition, all three cases demonstrated a similar pattern of demyelination of the middle root zones of the posterior columns in the spinal cord. Demyelination of the spinocerebellar tract was noted bilaterally.

Another member of the second family who also died with ALS was recently studied further; all the previously described neuropathologic changes were confirmed. But two new features were observed. One was the presence of ballooned anterior horn cells. The ballooning was due to accumulation of hyalin-like material within the cytoplasm of the affected neurons. Occasionally a more deeply stained central core, which resembled Lewy's inclusions, was observed (fig. 3). The cytoplasm was often occupied by a single large ball of material, but more frequently the inclusions appeared as multiple bodies. Some of the neuronal processes were also often distended with this material (fig. 3). The other unusual finding was the accumulation of amorphous material between the Purkinje cell layer and the granular layer of the cerebellum, and it was more readily seen in the region of the culmen.

Such an alteration has not been observed in other cases, and the nature of this material is not known.

In contrast to these cases, the neurologic and pathologic examination of an adult male whose brother and father also died of ALS revealed the classic picture of sporadic ALS.

3. *ALS on Guam*

Neuropathologic findings in ALS among the natives of Guam were documented 20 years ago by Zimmerman (2), who made anatomic diagnoses of two Chamorro cases during 1 month in 1945. Along with the development of extensive clinical investigation of Guam ALS since 1952 and 1953, neuropathologic features of Guam ALS were reported in detail by Arnold, Edgren, and Palladino (3) in 1953, Kurland and Mulder (4) in 1954 and Sayre (5) in 1957. These investigators confirmed the clinical diagnosis of ALS neuropathologically.

In late 1957, Malamud first recognized Alzheimer's neurofibrillary changes in the pigmented neurons of the substantia nigra and pontine tegmentum of Guam ALS cases which otherwise revealed the typical histopathology of ALS. However, there were no inflammatory changes. This observation led to subsequent careful examination of additional ALS cases from Guam with special attention to neurofibrillary changes. Analysis of 22 cases of ALS among the Chamorros on Guam was reported by Malamud, Hirano, and Kurland (6) in 1961. The histologic changes char-

acteristic of ALS were consistently observed in all 22 cases.

However, in all cases, Alzheimer's neurofibrillary changes were also present. The extent of neurofibrillary changes varied from case to case, but they were distributed in a characteristic fashion within the central nervous system. The involved areas were the hippocampus, especially the pyramidal cells in Sommer's sector and the glomerular formation of the hippocampal gyrus; frontotemporal cerebral cortex; various hypothalamic nuclei; substantia innominata; amygdaloid nucleus; periaqueductal gray matter including oculomotor nuclei; median raphe; substantia nigra; locus caeruleus; reticular formation of the brain stem; dorsal efferent nucleus of the vagus; motor cranial nuclei of the pons and medulla; dentate nucleus of the cerebellum; and intermediate gray matter of the spinal cord. Neurofibrillary changes were also found in the anterior horn cells of the spinal cord in seven instances. Although the intensity of neurofibrillary changes varied from case to case, it did not appear to correspond either to severity of motor neuron damage or to duration of illness. Granulovacuolar bodies were observed in 20 cases and were most prominent in the pyramidal cells of Sommer's sector.

Although neurofibrillary changes are considered as the usual companions of senile plaques, such plaques were observed in the hippocampus in only 3 of the 22 cases, and in these cases only 2 or 3 plaques were observed per section. Furthermore, in 4 of the 22 cases

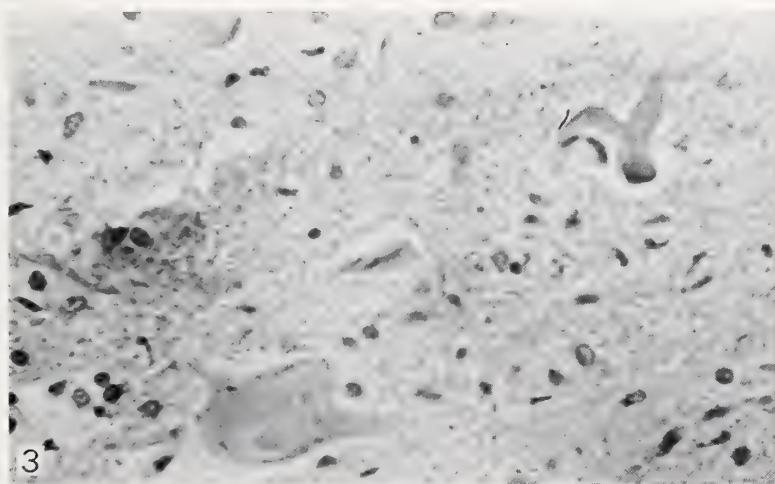


FIGURE 3.—Cervical enlargement of spinal cord. Three hyaline inclusion bodies in swollen anterior horn cell contain central cores. Distended neuronal process is seen (upper right corner). Patient had familial ALS with involvement of posterior column and spinocerebellar tract. (Hematoxylin and eosin; $\times 400$.)

there was distinct pathologic evidence of pallidonigral degeneration in addition to the changes of ALS.

These findings have been confirmed by further histopathologic investigation of 20 more recent cases of ALS from Guam. There are some interesting additional findings in these necropsy cases. The illness occasionally developed in a younger person whose clinical course might be longer than the average for all ages combined. For example, the age at death of the youngest patient was 28 years, after a 5-year course of the disease. Two patients died at ages 29 and 30 years, respectively, each after approximately a 4-year course. Neurofibrillary changes were evident even in these young patients, but here again senile plaques were observed infrequently in only two cases in the entire group. The pigmented neurons of the substantia nigra, locus caeruleus and other areas of the brain stem were usually affected but the severity of involvement ranged from macroscopic atrophy to only rare occurrence of neurons with altered fibrillary tangles. The neurofibrillary changes in the substantia nigra in the Guam cases of ALS are of particular interest because such changes are rarely observed in the familial or classic cases of ALS, although too few familial cases have been studied to draw any firm conclusions regarding this feature.

Neurofibrillary changes were not observed in the substantia nigra in the classic cases of ALS seen at Montefiore Hospital, New York (7). From more than 80 necropsies in ALS cases in the files at Montefiore Hospital, 39 brains and spinal cords were selected and examined by the same methods as those applied to the Guam cases. Although some neurofibrillary

changes were observed in the cerebral cortex from seven of the elderly patients and a small number of these changes were seen in the locus caeruleus in three instances, involvement of the substantia nigra was observed in only one case. As previously reported (7), this case was atypical both clinically and pathologically. In addition to the unusually long clinical course (11 years), neurofibrillary changes were widely distributed. Neurofibrillary changes were not found in the anterior horn cells, but intracytoplasmic concentric inclusion bodies remarkably similar to Lewy's bodies were observed (fig. 4).

4. *P-D Complex on Guam*

In earlier papers the clinical and pathologic features of the P-D complex were described (8,9). There were gross cerebral atrophy and depigmentation of the substantia nigra and locus caeruleus. A consistent microscopic finding was widespread neuronal degeneration of the central nervous system, with numerous neurofibrillary changes in affected areas. The detailed topography of Alzheimer's neurofibrillary changes, which had been reported previously (7) in the Guam cases of ALS, showed a remarkable similarity in the distribution of the altered neurons in the cases of P-D complex although the substantia nigra in the P-D cases tended to be severely involved. Numerous granulovacuolar bodies were also present in the pyramidal cells of Sommer's sector, and occasionally they were also seen in other regions. However, typical senile plaque formation, which is the most con-

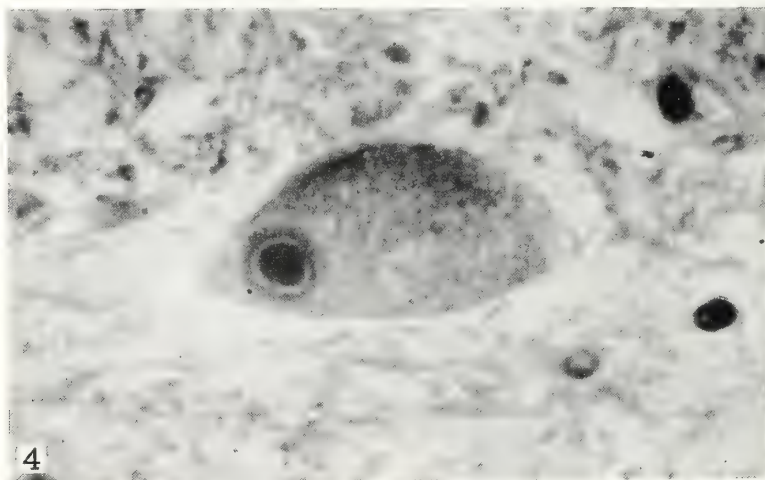


FIGURE 4.—Intracytoplasmic concentric inclusion body in anterior horn cell of spinal cord in atypical case of ALS. (Hematoxylin and eosin; $\times 1,100$.)

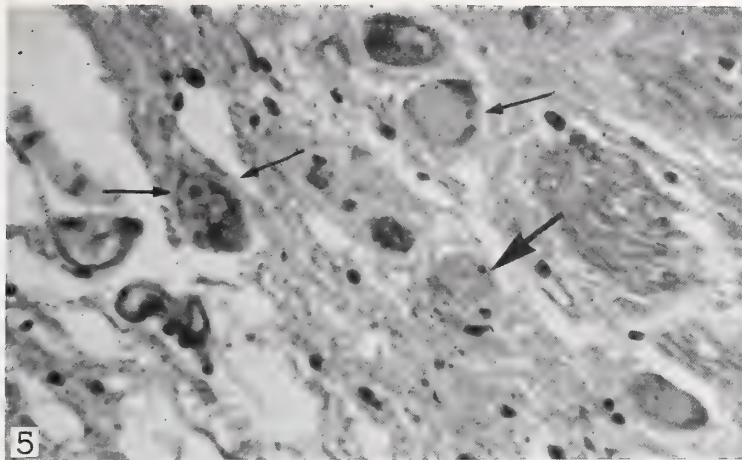


FIGURE 5.—Neurons with Lewy's bodies (thin arrows) and Alzheimer's neurofibrillary changes (thick arrows) in substantia nigra in a case of P-D complex on Guam. (Hematoxylin and eosin; $\times 400$.)

spicuous finding in classic Alzheimer's disease and senile dementia, were usually not observed.

Since these 17 cases were reported, material from 30 additional cases has been examined neuropathologically. Generally speaking, findings in these cases confirmed the observation of the previous report. However, several additional features have been intensively studied, and the observation of Lewy's bodies in the substantia nigra and the electron microscopic findings in Ammon's horn have been selected for a brief description at this time.

While Alzheimer's neurofibrillary changes were consistently found in the substantia nigra in the earlier series, Lewy's intracytoplasmic concentric hyaline inclusion bodies were not observed (8). However, in five of the 30 P-D cases recently studied, Lewy's bodies were found in some of the remaining neurons of the substantia nigra in proximity to neurons with neurofibrillary changes. Therefore, these two types of neuronal changes were occasionally observed within the same stained sections (figs. 5 and 6). Neuronal loss was the most conspicuous finding in these cases, how-

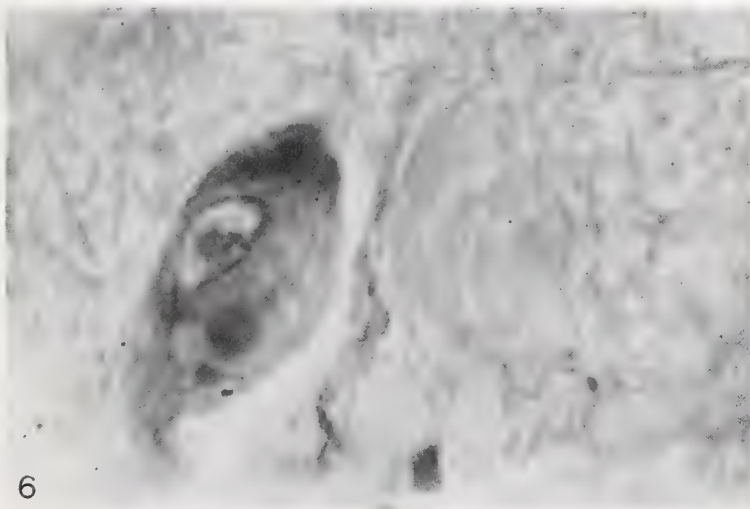


FIGURE 6.—Higher magnification of area similar to that shown in Figure 5. Neuron with hyaline inclusion (left) and mass of fibrillary tangle (right) are seen side by side. Patient had P-D complex. (Hematoxylin and eosin; $\times 1,000$.)

ever, and the number of neurons affected with neurofibrillary changes or Lewy's bodies was small. This observation is of some interest, for it has been reported that in paralysis agitans, Lewy's bodies are observed in "idiopathic" cases, whereas neurofibrillary changes are said to be more common in the "postencephalitic" cases (10-12). Although there are diverse reports on this subject (13), the simultaneous occurrence of both types of neuronal change is considered in the literature to be exceedingly rare (14, 15).

Since the pyramidal cells in Sommer's sector were the site of predilection for neurofibrillary changes (fig. 7), an effort was made to determine the electron microscopic characteristics in this area in seven cases from Guam. In order to obtain better preservation of necropsy tissue, the tissue blocks were fixed in chilled 5 percent glutaraldehyde solution in phosphate buffer at pH 7.4 at the time of necropsy. The blocks were sent from Guam to Montefiore Hospital by air mail. They were washed in chilled phosphate buffer at pH 7.4. With the aid of a dissecting microscope, Sommer's sector was removed and was cut into smaller fragments. These fragments were then immersed in Dalton's fixa-

tive for 1 hour, dehydrated, and embedded in Epon. Sections were cut on a Porter-Blum ultramicrotome with glass or diamond knives. They were stained with lead hydroxide or saturated uranyl acetate and examined with an RCA EMU 3F electron microscope.

Many of the neurons were filled with abundant neurofibrils which were composed of many neurofilaments. Individual neurofilaments were of indeterminable length, and showed no periodicity. They were apparently oriented in parallel rows in the longitudinal section. The cross-section view of the filaments showed a circular density (fig. 8) similar to those reported by Terry (16), Kidd (17), and Luse and Smith (18) in cortical biopsy specimens from cases of Alzheimer's disease.

In addition to the neurofibrils, more compactly arranged structures were observed. These structures were composed of numerous, very dense parallel filaments (fig. 9), sometimes seen in a circular (fig. 10) or herring-bone-like arrangement (fig. 11). The dense, parallel and compactly arranged filaments were approximately 100 Å wide (fig. 9). In some other areas, less electron-dense bands, with a suggestion of triple

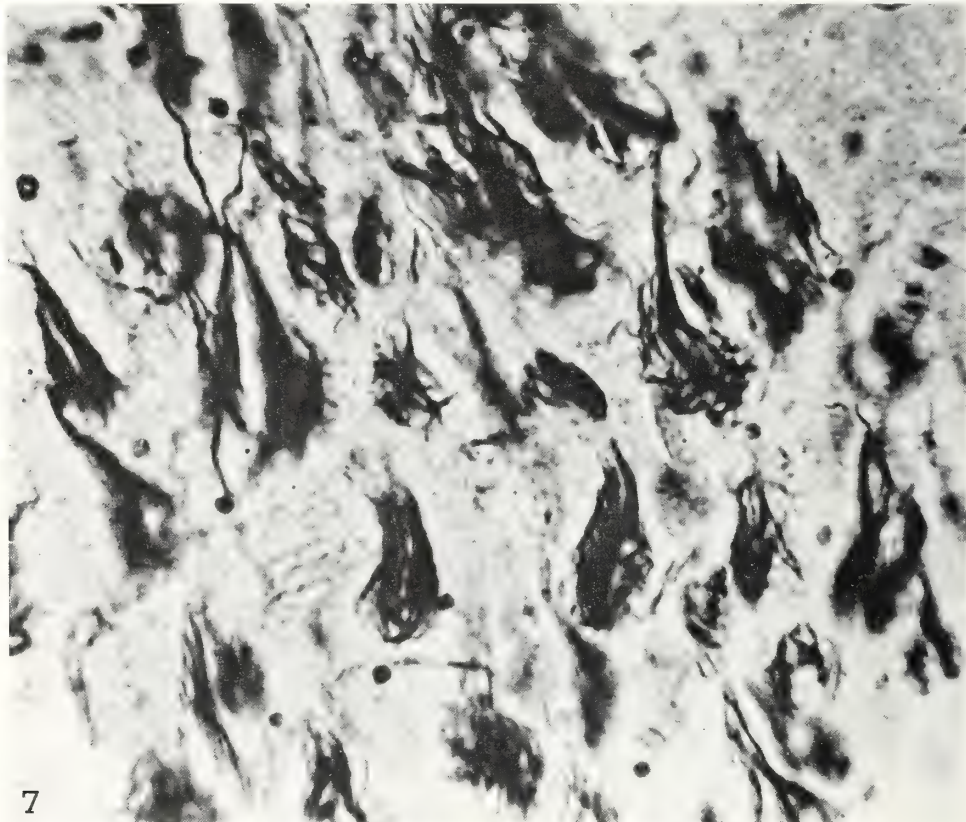


FIGURE 7.—Pyramidal cells in Sommer's sector show neurofibrillary changes in case of P-D complex on Guam. (Silver impregnation; $\times 600$.)

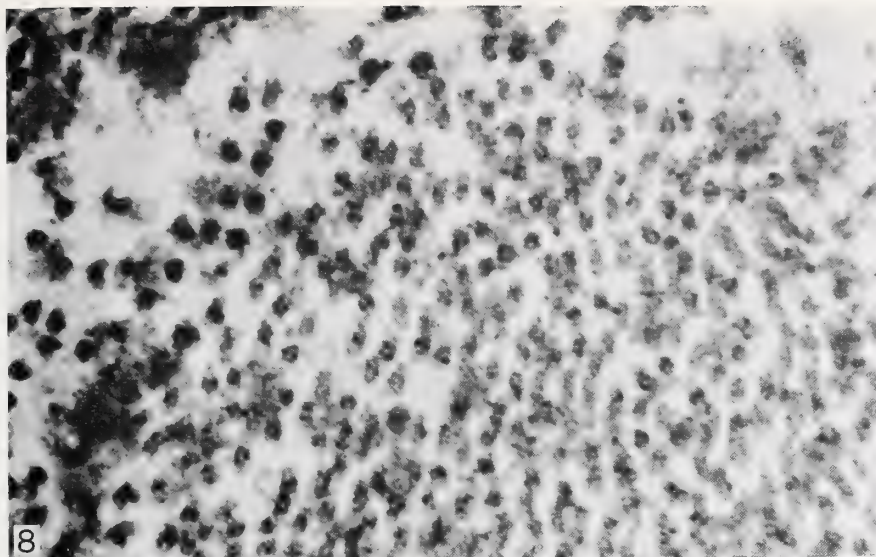


FIGURE 8.—Cross-section view of loosely arranged filaments show circular density in profile. $\times 160,000$. Figure 8 through 13 are electron micrographs of fibrillary structures from affected pyramidal cell layer in Guam cases. Sections were strained with uranyl acetate, lead hydroxide, or both.

density, were observed (fig. 9). These less electron-dense bands varied in width from about 120 Å to 200 Å. In general, triple density outlines appeared more clearly in the wider bands, which were usually distributed less compactly (fig. 12). In addition to the above-mentioned lines, another interesting pattern was observed. Many minute, electron-dense, round particles were arranged in a remarkably regular pattern (fig. 13). Each particle was approximately 100 Å in diameter and the particles were oriented with regular intervals in rows which were often separated by narrow electron-dense lines. These structures have apparently not been described before in the nervous system, and their nature is unknown.

5. *Preliminary Evaluation of Specimens with Eosinophilic Intracellular Inclusion Bodies and of Specimens in Experimental Transmission of ALS*

In 1962, Bunina (19), in the U.S.S.R., described intracellular inclusions in the motor neurons of the spinal cord and of the brain stem in two cases of "hereditary amyotrophic lateral sclerosis" and concluded that "their presence may have been due to a neurotropic virus." More recently (1963), Zil'ber and colleagues (20) of the U.S.S.R. reported experiments which were

interpreted as suggestive of the reproduction of ALS in monkeys that had received an intracerebral inoculum prepared from the spinal cord of persons who had died of ALS. They concluded that the experimental evidence strongly suggested that the human disease was of viral origin.

Through arrangements made possible by the U.S.S.R.-U.S.A. Scientific Exchange program, specimens were received from Professors Zil'ber and Bunina. These specimens were paraffin-embedded tissue blocks from the spinal cord of two human ALS cases and of two experimental monkeys.

The findings in these tissues blocks will be compared with those in ALS cases from Guam and ALS cases studied at Montefiore Hospital. Certain other cases in which myelopathy was present have also been restudied for comparison. Altogether five groups of cases were compared. In the following outline of material studied, groups A and B represent Professor Bunina's material, and the information that accompanied the 11 paraffin blocks received from him is placed in quotation marks.

MATERIAL STUDIED

Group A.—Two human ALS cases. (1) ALS. " * * * typical clinical picture of the classic form of the disease. The patient died November 13, 1959, at the age of 22." There were three Formalin-fixed



FIGURE 9.—Parallel and compact arrangement of electron-dense and less electron-dense bands. $\times 99,000$. See legend to figure 8.

blocks: Medulla oblongata (block No. 1), spinal cord at C7 (No. 2), and spinal cord at L3 (No. 3). (2) "Familial ALS, bulbar form. The patient died October 16, 1955, at the age of 61. Duration of the disease—1½ years." Three Formalin-fixed paraffin blocks represented the spinal cord at C6 (No. 4), T1 (No. 5), and L3 (No. 6).

Group B.—Two monkeys inoculated with medullary extract from patients who died of ALS. "These monkeys were affected with paresis of the extremities: the amyotrophies agreed with the increase of the tendon reflexes." (1) Monkey "Silky." Two Formalin-fixed blocks were from the brain stem at the "level of commissure (No. 7)" and from the spinal cord at L3 (No. 8) respectively. (2) Monkey "Mys." Two Formalin-fixed blocks were from the spinal cord at C6 (No. 9) and the lower lumbar region (No. 10) respectively. An additional block, fixed in "Carn...t's

solution," (?) was from the spinal cord at C7 (No. 11).

The 11 blocks from Professor Bunina were cut at thicknesses of 6 and 15 microns and stained with hematoxylin and eosin, periodic acid-Schiff stain, Luxol Fast Blue-neutral red, cresyl violet, phosphotungstic acid hematoxylin, Mann's stain, and silver impregnation.

Group C.—The spinal cords from the 21 Guam cases (12 ALS and 9 PD complex) were reexamined for special comparison with the U.S.S.R. material.

Group D.—Material from five sporadic and one familial case of ALS from the files of the Montefiore Hospital was reexamined.

Group E.—Paraffin-embedded, hematoxylin-and-eosin-stained sections of the affected anterior horn cells were also reviewed in the following cases: three cases of familial ALS with posterior-column involvement (1), two cases of Jamaica neuropathy (21), a case of

subacute necrotizing myelopathy (Foix and Alajouanine), and a case of Guillain-Barré syndrome from the files at Montefiore Hospital.

RESULTS

Group A.—The histologic features of the two cases of human ALS were identical with those of classic ALS, as Professor Bunina described. The principal morphologic features were a paucity of motor neurons among the anterior horn cells, extensive demyelination of the pyramidal tracts, and severe involvement

of the anterior roots. In neither case was there demyelination of the intermediate portion of the posterior columns or spinocerebellar tracts. There was no accumulation of the hyalinized material that had been observed in the perikaryon and processes of the affected motor neurons in one of our families with hereditary ALS. Intraganglionic fibrillary alterations such as those frequently found in ALS and PD complex on Guam were not observed.

Bunina had described and illustrated an eosinophilic intracytoplasmic inclusion body in the report. These

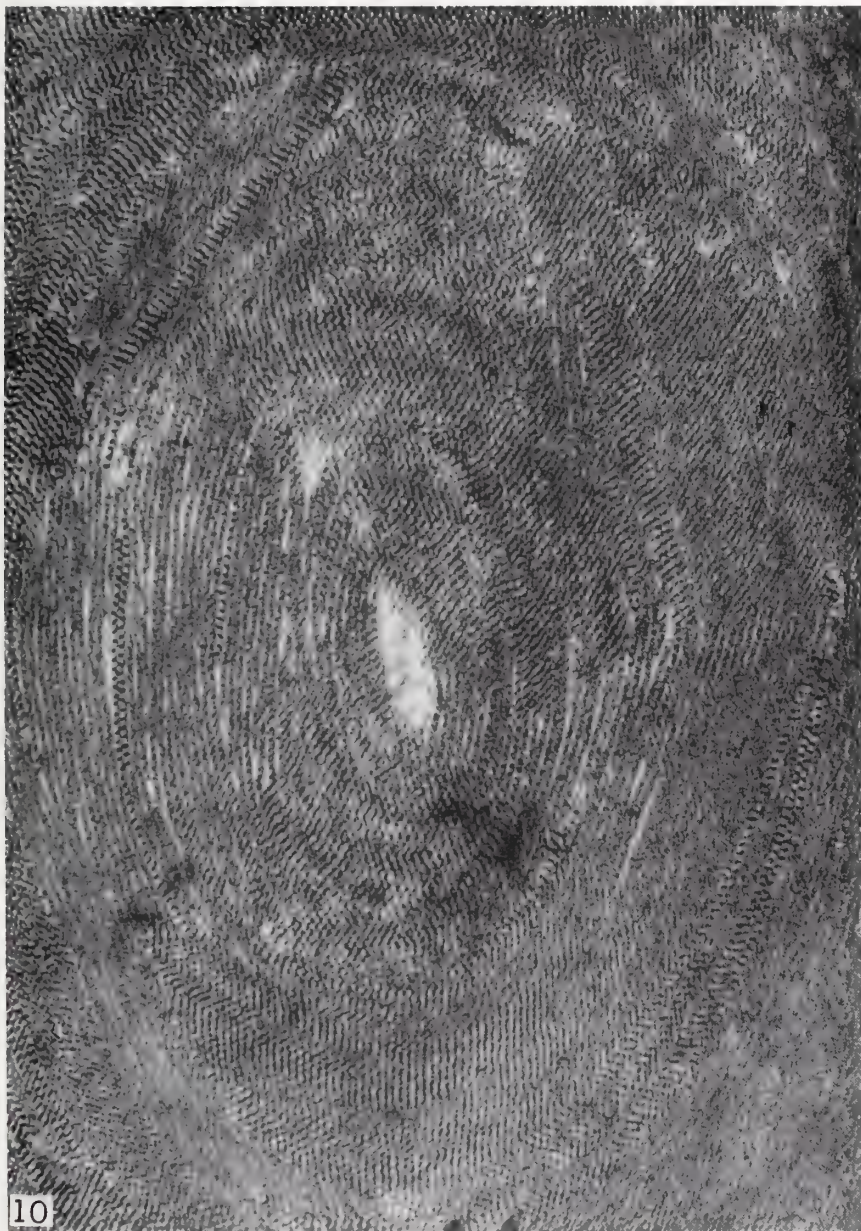


FIGURE 10.—Ring-like arrangement of fibrillary structure. $\times 67,000$. See legend to figure 8.



FIGURE 11.—Herring-bone-like arrangement of fibrillary structure. $\times 96,000$. See legend to figure 8.

were observed in the case of familial ALS (block 6) in sections taken from the L3 segment of the spinal cord and stained with hematoxylin and eosin and Mann's stain and in sections taken from the C6 and T1 segments of the cord and stained with hematoxylin and eosin. They were not observed in sections from any of the other blocks.

The inclusions observed had the following morphologic characteristics: they were minute, round, bright-pink bodies within the perikaryon of the altered neurons in the region of the anterior horns. The number

of bodies varied from one to many, but most often two to seven. The size of the individual bodies appeared to vary, but most of them were smaller than one quarter of an erythrocyte, being approximately 1 to 3 microns in diameter. Larger inclusions had halos around them, but the smaller bodies, owing to their minute size, were hard to differentiate from optical artifacts. Neurons containing these bodies were relatively rare—at most, three neurons per section 6 microns thick. The normal pattern of Nissl substance was often no longer observed. Powdery basophilic

substance was usually seen in the periphery of the swollen perikaryon, and the main cytoplasmic content seemed finely granular or liquefied. These bodies stained dark red with Mann's stain. However, because of the small size and rarity of these bodies, it was extremely difficult to determine their special staining reaction with periodic acid-Schiff stain, silver impregnation, phosphotungstic acid hematoxylin, Nissl stain, and Luxol Fast Blue-neutral red stain, and it was practically impossible to identify the inclusions when such stains were used. The bodies seen in sections stained with hematoxylin and eosin were not doubly refractile when polarized light was applied. The inclusion bodies were not found within the nucleus, the processes of neurons, or the glial cells.

Group B.—Although specimens from normal monkeys were not available for comparison, stained sections from the experimental monkeys seemed to reveal no changes identical with those in the human ALS described above. Definite demyelination of pyramidal tracts, significant loss or changes in motor neurons, and atrophy of anterior roots were not recognized in the sections from monkey "Silky." Fibrillary astrocytic processes were prominently noted in sections from C7 of monkey "Mys" that were stained with phosphotungstic acid hematoxylin. In the sections stained by the Nissl method there was increased satellitosis. There was also pallor of Goll's tract. However, Formalin-fixed sections from C6 and the lumbar level did not reveal significant gliosis.

Attempts to find the eosinophilic inclusion bodies were unsuccessful in these prepared sections from

the experimental animals. Perivascular cuffing or other inflammatory changes were not seen in this material.

Groups C, D, and E.—Identical or similar eosinophilic inclusion bodies were observed in 10 altered anterior horn cells from two cases of ALS from Guam (more than 100 stained sections were examined in these two cases). They were seen in the cervical cord in one case (fig. 14) and in the cervical and lumbar cord in the other (fig. 15). They were also observed in nine anterior horn cells of the cervical and lumbar cord in two cases of ALS from the Montefiore Hospital (fig. 16) (more than 170 sections were examined in these two cases). In neither of the latter cases was there a family history of ALS. In one additional case of familial ALS in New York, a few eosinophilic inclusion bodies were noted in several neurons of the spinal cord. The inclusion bodies have not been found so far in the other cases examined.

COMMENT

The major point from the work of Zil'ber and Bunina and their colleagues was that a disease similar to ALS developed in monkeys 1 to 3 years after they had received intracerebrally emulsions of spinal cord and brain from persons who had died of ALS. They stated that the illness in monkeys was similar to the human disease in its clinical and pathologic features.

The paraffin-embedded blocks available to us from the two monkeys were either insufficient or nonrepresentative, so that the present findings cannot sup-

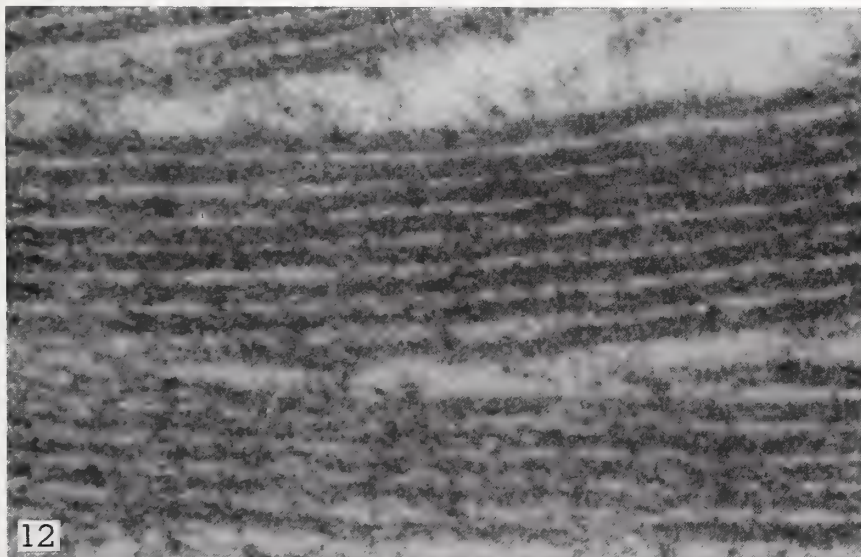


FIGURE 12.—Loosely distributed, less electron-dense band shows triple density outline. $\times 180,000$. See legend to figure 8.

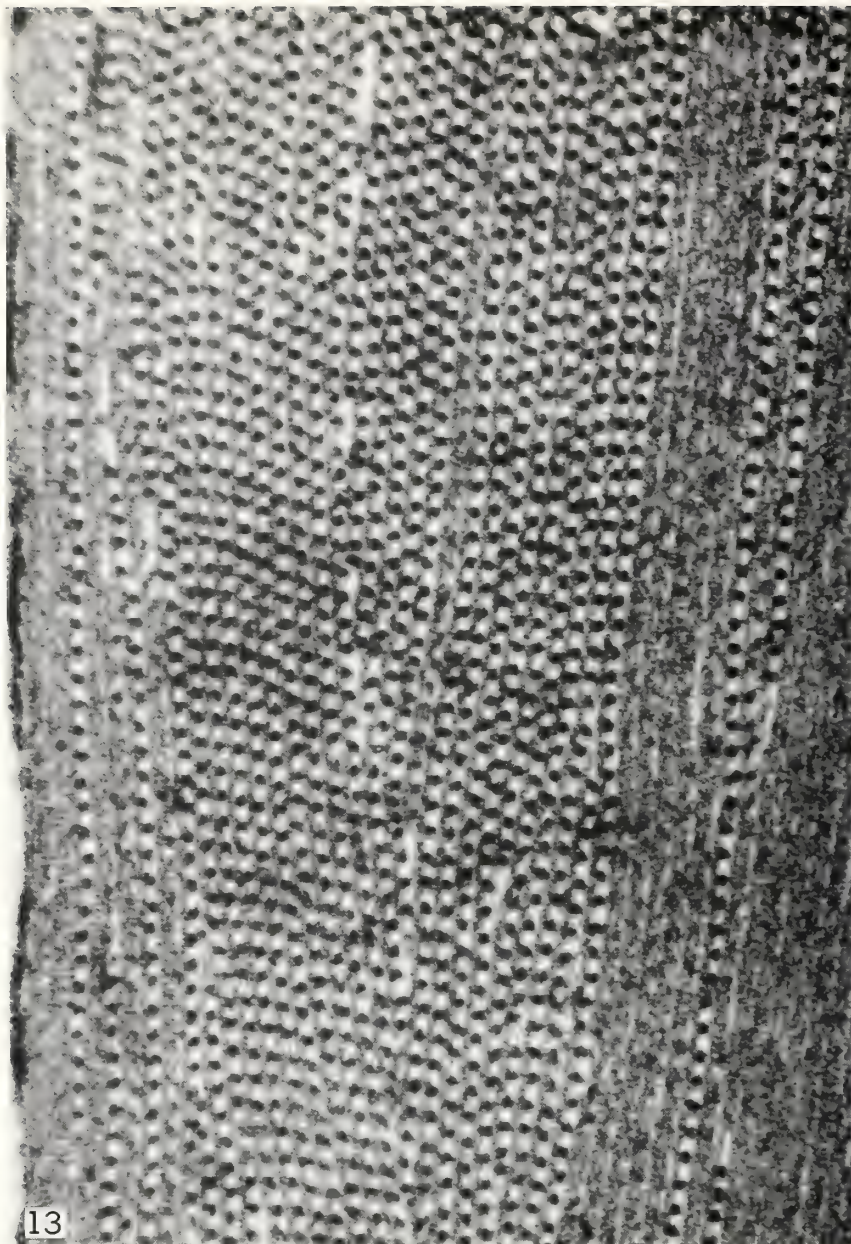


FIGURE 13.—Many bits of minute, electron-dense, round, particle-like substance are arranged in a remarkably regular pattern on a fine electron-dense line. $\times 186,000$. See legend to figure 8.

port the statement of these investigators. The characteristic histologic changes of ALS, so clearly observed in their human cases, were not found in the sections prepared from the experimental monkeys. For further investigation of the delicate alterations, additional frozen sections for Sudan staining and celloidin blocks for better myelin preservation and cell-population studies would be necessary. The need for control

animals in the Soviet experiments has already been recognized.

From the standpoint of histologic alterations, the key point in their report is their observation of intracytoplasmic eosinophilic inclusions in the affected anterior horn cells and cranial nerve nuclei in "hereditary ALS" and in sporadic cases in which life was maintained beyond the usual duration by a respirator.

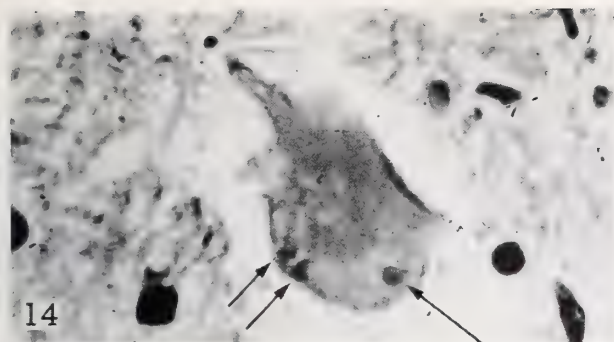


FIGURE 14.—Three intracytoplasmic inclusion bodies (arrows) in altered anterior horn cell of cervical spinal cord in a case of ALS on Guam. (Hematoxylin and eosin; $\times 980$.)

We confirmed the presence of these bodies in one of their "familial ALS" cases but not in the other and not in the experimental animals, although such bodies were reported by the investigators originally (19, 20).

These intracytoplasmic eosinophilic inclusion bodies are different from any inclusions previously studied by us. They were clearly different from hematoxylinophilic granulovacuolar bodies. They were not the intranuclear eosinophilic inclusions reported in viral or suspiciously viral diseases including herpes simplex encephalitis, subacute inclusion encephalitis (Dawson), subacute sclerosing leukoencephalitis (van Bogaert), acute necrotizing encephalitis (van Bogaert), cytomegalic inclusion disease, acute poliomyelitis, acute necrotic encephalitis, and progressive multifocal leukoencephalopathy, in noninfectious conditions such as hepatocerebral diseases, and in elderly persons (Marinesco) (22–24). They were much smaller, showing different morphologic features from those of Alzheimer's neurofibrillary changes or Lewy's concentric hyaline inclusion bodies. They differed from the numerous eosinophilic and carmine-positive granules observed within the swollen axons in the affected sub-

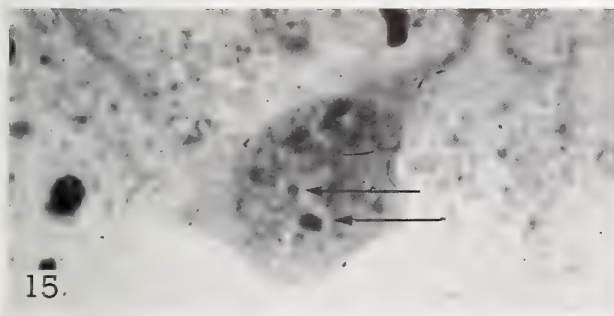


FIGURE 15.—Two inclusion bodies (arrows) in anterior horn cell of lumbar spinal cord. ALS on Guam. (Hematoxylin and eosin; $\times 980$.)

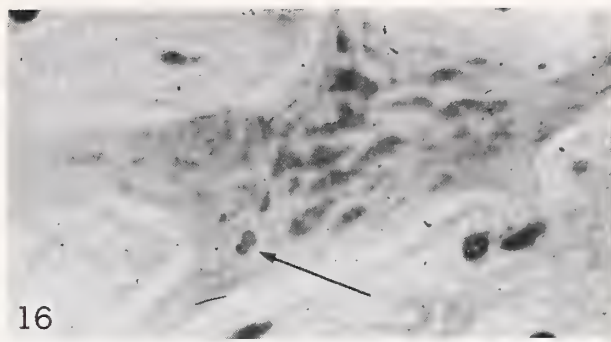


FIGURE 16.—Two inclusion bodies (arrows) in anterior horn cell of cervical cord show halo around them in a case of sporadic ALS from New York. (Hematoxylin and eosin; $\times 980$.)

stantia nigra, Goll's nucleus and periaqueductal gray matter in some cases of parkinsonism, in PD complex, or other conditions (25).

In view of Bunina's report on familial ALS, the finding of such eosinophilic inclusions in some of the altered but remaining anterior horn cells of two Guam cases of ALS is of considerable interest. Our Guam cases are generally considered familial although the relative genetic and nongenetic influences on familial aggregation are still open to debate. Furthermore, neither of the Guam patients had been maintained in respirators. The likelihood that these inclusions are nonspecific for different forms of ALS is denoted by the fact that they have now been observed not only in familial and sporadic cases in the U.S.S.R. but also in sporadic cases elsewhere, in the one of four familial cases in which there were no posterior column changes, and in two of the Guam cases as well.

Are these bodies specific for ALS? So far it has not been possible to find such inclusions in the affected anterior horn cells of various other diseases, and this supports the hypothesis that they are associated with ALS. However, this preliminary study was done with only a limited number of sections from a rather small number of cases. Without a systematic and extensive investigation, it would not be proper to draw any firm conclusions. Furthermore, these bodies are often so minute and so few that it would be difficult to ascertain their absence.

Finally, intracytoplasmic eosinophilic inclusions known as Negri and lyssa bodies have been found in the pyramidal cells of the hippocampus, in the Purkinje cells of the cerebellum, and in the motor neurons of the spinal cord. The name "lyssa bodies" was given to "simple acidophilic, cytoplasmic inclusions with no internal structure. But inclusions of this type are not so characteristic of rabies as Negri bodies, as similar

acidophilic droplets may be seen in nerve cells in a variety of degenerative conditions which are not related to virus infections" (24).

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DISCUSSION

MARGOLIS: I would like to ask about the implications of the observations regarding the neurofibrillary change. If we take a group of patients dying at

about the age of 80, and if we look very carefully, we will find such changes in practically every brain. I do not think it is right to use the granulovacuolar change, senile plaque or neurofibrillary change, and equate these with insanity, or with a known post-encephalitic disease. These may be important leads and if the lesions are established as being caused by a virus, then they are terribly important, but I think we have to recognize that we cannot do that now.

HIRANO: I do not know, nor did I mean to imply, whether or not these neurofibrillary changes are associated with a virus. In addition, I can only point out again that some of the Guam patients showed these changes as young as 28 or 29 years old.

POSKANZER: As this question has been brought up, one can also consider senility as a viral disease. It is possible that these manifestations represent viral infection. We have had a series of five cases with amyotrophic lateral sclerosis and presenile dementia. The chance of these two diseases occurring together is very small. It looks as if there may be some association between these two diseases which we have not recognized before.

BRODY: To relate this association to the Guam cases, does the evidence suggest that ALS is related to PD etiologically; and does it further suggest that the ALS on Guam is a unique disease and different from the ALS in the rest of the world, or are we just be-

ginning to get into a whole spectrum of disease which has different geographic manifestations?

KURLAND: We have been emphasizing in this presentation some of the differences. We must bear in mind that, clinically, the ALS seen on Guam in its earlier stages would be extremely difficult to differentiate from that seen elsewhere. However, the recent studies of some of our associates revealed that when the patients were examined regularly and thoroughly about 12 percent showed evidence of dementia or extrapyramidal disease near the termination of their ALS; whereas about one third of those who started with parkinsonism-dementia developed amyotrophy before they died. Perhaps the same intensive follow-up is necessary first for a series of classical ALS cases; nevertheless, I suspect that on Guam we have observed a spectrum, with several clinical variants, of a single disease. Whether this is due to our unusual opportunity to observe the total population with respect to neurologic diseases, and we are therefore seeing the full spectrum of ALS, or whether there is a distinct form of the disease on the island remains to be seen. However, there are many similarities of the Guam ALS with that seen elsewhere so that any clarification in the picture in the Mariana Islands will, I feel certain, shed some light on the ALS problem in general.

Attempts to Demonstrate a Transmissible Agent in Kuru, Amyotrophic Lateral Sclerosis, and Other Subacute and Chronic Progressive Nervous System Degenerations of Man

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This communication is a summary of our program of study on the possible infectious etiology of slowly progressive degenerative human central nervous system diseases of a subacute or chronic nature. In general, it presents negative data, after as long as 2½ years of observation, on attempts at virus isolation and experimental transmission, particularly in primates, of kuru and amyotrophic lateral sclerosis (ALS). Moreover, it briefly describes our experiments, after 4 to 12 months' observation, on subacute inclusion encephalitis of the Dawson type and attempts to transmit serially and characterize a motor neuron disease in primates that has been described by investigators in the U.S.S.R. as an isolate from a human case of ALS. Finally, preliminary findings are presented and discussed on the isolation of a cytotoxic agent for primary and stable renal cell cultures of *Cercopithecus aethiops* from the lymph node of a human suspected of having Aleutian mink disease. Because of the increasingly frequent suggestions that a transmissible agent may be involved in the pathogenesis of these diseases (1-14), this progress report on experiments, which will not be terminated before 5 to 6 years, is in order.

We believe that it is important at this time to re-emphasize the basis for our program as earlier presented by Dr. Gajdusek in the opening session of this workshop-symposium in order to stress the application of the concept of slow virus infections in investigation of the diseases under study. Thus, at the time of medical discovery of the kuru focus among the Fore people and their neighbors in the highlands of Eastern New Guinea, the possibilities of acute infectious, postinfectious, and of chronic persistent infectious etiology, as

well as an infection-stimulated hypersensitivity state were all considered (15-16). The cause of this unremitting and unaltered plague on some 30,000 Melanesian natives, still causing today over half the mortality in the region of highest incidence, has remained unsolved.

Early in 1957 small laboratory animals, including chick embryos, mice, rats, guinea pigs, and rabbits were inoculated with suspensions of brain tissue from kuru patients in an attempt to transmit the disease experimentally, or to propagate a microorganism associated with it. Neuropathological studies of patients had failed to elicit any definitive histopathological lesions in the central nervous system (CNS), such as are commonly associated with viral infections. Furthermore, clinical laboratory studies of cerebrospinal fluid specimens from kuru patients did not elicit findings such as pleocytosis or elevated protein values common, at least in a high proportion of cases, to all known CNS viral infections of man. Patients failed to show febrile reactions at any stage of the disease, no consistent change in erythrocyte sedimentation rate or in the peripheral blood picture was found, and no episode of contact infection was recorded epidemiologically. Thus, infectious etiology seemed unlikely. Nevertheless, brain tissue obtained from kuru patients taken shortly after death was preserved in buffered glycerin or by ice refrigeration and flown to Melbourne, Australia, where isolation attempts were initiated. Some of these specimens were subsequently shipped under dry ice refrigeration to the National Institutes of Health, Bethesda, Md., where additional isolation studies were conducted utilizing small labora-

tory animals, embryonated hens' eggs, and a variety of tissue and cell cultures prepared from human, animal, and avian origins. These early studies failed to elicit any recognizable or recoverable pathogenic microorganisms during the 3 months that the animals remained under observation.

More recently, however, Hadlow (3) reported that a similarity exists between the neuropathological lesions in sheep with scrapie disease and humans with kuru. Amplification of the detailed neuropathological findings of these two diseases will be presented during this workshop by our collaborators, Mrs. Elisabeth Beck and Prof. P. M. Daniel (17), of the Department of Neuropathology, Maudsley Hospital, Institute of Psychiatry, London. The observations made by Hadlow led us to re-evaluate our isolation procedures and laboratory techniques and to reconsider the possibility that an etiological agent for kuru might be a slow or latent virus with, perhaps, a genetically determined susceptibility, or perhaps, even more speculatively, a temperate virus actually genetically transmitted, acting somewhat like a temperate bacteriophage. Indeed, because of the unusual heat stability and alleged genetic influence on transmission in flocks of sheep, the suggestion had often been made that the scrapie agent might be denuded nucleic acids originating from the host genetic mechanism itself, and not a complete virus particle as commonly encountered in virology (18) (the extent to which infectious nucleic acids do occur and the associated problems in virology are well reviewed by Herriott (19)). Although such a postulate would help to explain the lack of detectable antibody in scrapie disease, more detailed study of heat inactivation of the agent and its enzyme sensitivity, including the failure thus far to inactivate its infectivity with desoxyribonuclease and ribonuclease, and other rather more conventional virus properties of the pathogen, have convinced most workers that the scrapie agent is probably not denuded nucleic acid

but more like an orthodox animal virus (20, 21). Considerable data in support of the viral nature of the scrapie agent has been accumulated by us in our laboratory, as well as by other workers in this field, during the past 2 years and these findings will be presented later on during this workshop-symposium.

Beginning in 1962 long term studies aimed at observing primates for 5 years or more, and smaller animals and tissue cultures for as long as possible, were initiated in our laboratories. Table I summarizes the isolation studies now in progress on specimens of CNS tissues from patients who had died of kuru. Although only one chimpanzee (*Pan satyrus*) has, in general, been inoculated with the material from a single patient, other primates, rhesus (*Macaca rhesus mulatta*), cynomolgus (*Macaca cynomolgus iris*), African green (*Cercopithecus aethiops*) monkeys, and the Barbary ape (*Simia sylvania*), have been inoculated in groups varying from a few to a dozen with inoculum from a single patient (see table II). In addition, groups of large numbers of newborn and weanling mice of several strains, hamsters, guinea pigs, and primary and stable tissue and cell culture lines have been inoculated with serially diluted central nervous system (CNS) tissue suspensions (in phosphate buffered saline, pH 7.4, or appropriate cell culture media) prepared immediately before inoculation from freshly thawed tissue. Most primates have received inocula of 0.2 ml. intracerebrally and 0.2 ml. intravenously.

As may be noted in table II, the earliest of these long term studies was initiated in small laboratory animals inoculated in October 1962. In January 1963, a 10 percent suspension of brain tissue from the right temporal region of the brain, obtained 4½ hours after death from a patient who had died of kuru, was inoculated intracerebrally and intravenously into a young (less than 6 months of age) rhesus monkey; this suspension was also inoculated into small laboratory ani-

TABLE I.—Attempts To Isolate in Primates a Transmissible Agent From Kuru

Geographic area, syndrome and test animals	Number of cases on study	Specimen for inoculation	Maximum duration of experiment (months post inoculation) ¹	Primates		Total number of nonspecific deaths during the course of experiment to date ²
				Total number	Number of species	
New Guinea:						
Kuru—inoculated primates	7	CNS	30 months	76	5	15
Controls—uninoculated primates caged with inoculated animals		none	21 months	15	3	2
Totals	7		Range, 16–30 months	91	5	17

¹ As of July 1965.

² Nonspecific deaths refer to animals whose deaths have been

directly attributable to severe parasitism, bloody diarrhea and dehydration; no CNS involvement has been observed.

TABLE II.—Attempts To Isolate a Transmissible Agent From Kuru

Patient identification	Specimen for inoc- ulation	Method of pres- ervation ²	Experimental host range by date of inoculation					Other mammals or avians	Tissue and cell culture	
			Primates							
			Chimpanzee	M. rhesus	M. cynomolgus	C. aethiops	S. sylviaia			
KURU:										
Eiro	CNS ¹	CO ₂	September 1963	January 1963	September 1963 January 1964	September 1963 January 1964	October 1962	November 1962		
Enage	CNS	CO ₂	August 1963	August 1963	September 1963	September 1963 January 1964	August 1963	August 1963		
Kigea	CNS	CO ₂	August 1963	August 1963	September 1963 August 1963	August 1963 January 1964	August 1963	August 1963		
Kigea	Serum ³	CO ₂					January 1963			
Kageinaro	Serum	CO ₂					January 1963			
Asago	Serum	CO ₂					January 1963			
Moba	CNS	CO ₂	September 1963		September 1963 January 1964	September 1963 January 1964	October 1962	November 1962		
Kariwani	CNS	N ₂	November 1963	November 1963			February 1964	February 1964		
Igierakaba	CNS	N ₂	February 1964	February 1964			February 1964	February 1964		
Kabuinampa ⁴	CNS	N ₂	February 1964	February 1964			February 1964	February 1964		

¹ Brain tissue used for inoculum was in all cases obtained at autopsy within a few hours of death.

² CO₂ = dry ice; N₂ = liquid nitrogen.

³ Acute blood specimens from Kigea and two other kuru patients inoculated into mice in 1962 and 1963.

⁴ Viable cells from N₂ preserved CNS tissue have been cultivated in vitro continuously since Sept. 21, 1964.

imals and tissue cultures. Although there have been some nonspecific deaths amongst the inoculated groups of animals, after 26 to 33 months of close observation none of these animals have developed any evidence of neurological disorders, nor has a viable agent been isolated in them; tissue culture studies likewise revealed no cytopathological effect or evidence of a chronic carrier state during the long period that we were able to maintain and observe the cultures.

As the scope of our program became enlarged and intensified in early 1963, greater emphasis was placed on the selection, preservation, and processing of specimens, on the range of experimental hosts employed, and on improved virological and tissue culture procedures for successful isolation. Thus, for example, brain and visceral tissue specimens have been obtained from kuru patients in New Guinea within 2 hours of death. As shown in table II, these tissues were immediately frozen in liquid nitrogen and shipped by air in liquid nitrogen to the National Institutes of Health where portions were rapidly thawed, ground to 20 percent suspensions in phosphate buffered saline solution (pH 7.4) and inoculated into test animals, mainly primates. Specifically, 6 chimpanzees, 32 rhesus, 25 cynomolgus, 10 African green monkeys, and 2 Barbary apes have been observed for from 17 to 30 months after inoculation with kuru brain material without developing signs of disease. In all, CNS tissue from 7 different kuru patients have been used in these primate inoculations (see tables I and II). These inocula have also been put into the following tissue culture lines: routinely, primary and stable rhesus kidney, BS-C-1 stable green monkey kidney, human amnion, Chang liver, chick embryo fibroblast, mouse embryo, Hep-2, L-cells and HeLa; on occasion into human embryo kidney, primary pig kidney, and pig choroid plexus. No cytopathogenic effect has been observed. In early September 1964, tissue culture explants were made from CNS tissue preserved in liquid nitrogen, taken from the kuru patient Kabuinampa. Viable cells were recovered from these CNS tissues which have now been carried in primary state for 11 months. Though these cultures have been subcultured, no additional characterization of these cells has been made to date.

We have, furthermore, been actively aware of the possibility that slow growing, transmissible agents may be involved in the pathogenesis of a wide range of chronic progressive neurological diseases. Among these the focus of amyotrophic lateral sclerosis (ALS) and the related parkinsonism-dementia syndrome (PD) in extremely high incidence in the Chamorro popula-

tion on the island of Guam, discussed at this symposium by Drs. Kurland and Hirano (22, 23), is of particular interest (24, 25). In these indigenous Micronesians ALS attains a prevalence some one hundred times greater than that observed in the United States, Europe, or Japan. We are searching for a possible transmissible agent in these diseases also; brain tissue from the United States and Guamanian ALS patients and Guamanian parkinsonism-dementia patients have been collected, preserved, and processed as has been described for kuru. Table III summarizes the numbers and types of cases under study. Thus far, we have inoculated 1 chimpanzee and 56 monkeys of several species with either suspensions of brain tissue or disrupted blood clots from 11 clinically diagnosed ALS cases; brain tissue from 2 cases of parkinsonism-dementia; and a suspension of pooled brain tissue from 2 ALS and 2 PD patients on Guam. As noted in tables III and IV, primates and other experimentally injected animals have been observed for from 9 to 23 months and no illness attributable to the inoculation has appeared amongst them.

In 1963, Zil'ber et al. reported the successful reproduction of a syndrome like amyotrophic lateral sclerosis in *Macaca rhesus* monkeys injected with extracts of the medulla oblongata and spinal cords of persons who had died of the disease. A monkey injected in 1956 was observed 1 year later to have developed hypertrophy of the muscles of the left forearm and hand, exaggeration of tendon reflexes in the hind limbs and loss of weight; these signs became more manifest during the ensuing 18 months at which time (2½ years after inoculation) the animal was sacrificed and a homogenate of its spinal cord injected intracerebrally into two monkeys. Both second passage monkeys were reported as having developed motor neuron disease. One of these animals, Pean N 3098, was sacrificed 4 years and 10 months after inoculation and specimens of brain and cord were brought to our laboratory from the U.S.S.R. in dry ice by Dr. L. A. Zil'ber. These tissues were homogenized to a 20 percent suspension and injected into rhesus monkeys before and after filtration and in combination with undiluted serum from ALS patients (see table V). During the 11 months since their inoculation one monkey, injected with 220mμ millipore filtrate, developed severe parasitism, diarrhea and dehydration and died; all other monkeys have remained normal in appearance. Of course, confirmation of the reported transmission of amyotrophic lateral sclerosis from man to primate is essential and, furthermore, it cannot yet be established that a transmissible agent causing motor disease in

TABLE III.—Attempts To Isolate in Primates a Transmissible Agent From Amyotrophic Lateral Sclerosis (ALS) and Parkinsonism Dementia (PD)¹

Geographic area and syndrome	Classification	Specimens inoculated	Number of cases	Maximum duration of experiment (months post inoculation) ²	Number of primates over total number of species on test	Number of nonspecific deaths to date ³
GUAM:						
ALS	Familial	CNS ⁴	4	21 months	26/4	8
PD	Familial	CNS	2	23 months	16/3	3
ALS-PD pooled ⁵	Familial	CNS	4	11 months	2/1	0
U.S.A.:						
ALS	Sporadic	CNS ⁶	1	9 months	5/1	1
ALS	Sporadic	Blood clots	5	7 months	2/1	0
ALS	Familial	Blood clots	1	11 months	1/1	0
U.S.S.R.:						
ALS	Sporadic	CNS (2d ⁷ passage monkey)	1	11 months	8/1	1
Uninoculated controls				9 to 23 months	14/4	2

¹ Parkinsonism dementia syndrome on Guam associated with amyotrophic lateral sclerosis.

² As of July 1965.

³ Majority of deaths occurred in poorly conditioned animals recently received from the jungles; deaths were attributed to parasitism, diarrhea and related dehydration.

⁴ Brain tissue used as inoculum was obtained from the

primates is the etiological agent of motor neuron disease in man. Indeed, it should be noted that in a recent paper it was reported that neither clinical nor pathological localization of the disease in monkeys has been definitely established (26). During these sessions we have heard Dr. Hirano report that he was unable to observe microscopic changes resembling those of ALS in numerous sections of the brain and spinal cord of monkeys inoculated with the ALS materials in the U.S.S.R. (23). Nevertheless, we are committed to extensive investigations on the existence and nature of the agent.

The data shown in table VI further summarizes our program in progressive degenerating diseases. At this time, since the topic will be discussed later on during these sessions, mention will only be made that we have injected monkeys with an established strain of scrapie virus, and these animals have exhibited no evidence of disease during the 11 months they have been under observation. On the other hand, the virus causing Aleutian disease in mink has been suggested as the causative agent of a case of encephalitis in man (9). We have recently received specimens from an ambulatory patient of a Veterans Hospital who is suspected of having Aleutian mink disease. Monkeys inoculated with suspensions of these specimens have not developed clinically recognizable signs of disease during the 8 months they have been under observation. In contrast, when suspensions of a surgically biopsied lymph

human cases at autopsy within a few hours of death.

⁵ Tissues from these patients pooled, homogenized, and inoculated into animals as indicated.

⁶ Viable cells from tissues (CNS) obtained at post mortem have been cultured in vitro continuously since Oct 2, 1964.

⁷ For passage history see Zil'ber et al., Bull. World Health Org., 29, 449 (1963).

node from the patient were inoculated into primary African green monkey kidney cell cultures and BS-C-1, a stable African green monkey kidney cell culture, a slowly progressive cytotoxic effect was observed. This cytotoxicity has been shown to be transmissible on serial passage in these cell lines and, more recently, produce a similar cytotoxic effect in primary hamster kidney and rhesus monkey kidney cell cultures both before and following filtration through a 220m μ millipore filter. In primary African green MKC cultures the infectivity titer has ranged from 10^{-4.2} to 10^{-7.8} TCID₅₀/0.2ml. It should be noted that Miss Nancy Rogers (27) of our laboratory has also isolated an agent from the lymph node and urine of the patient that produces a cytotoxic effect in BS-C-1 cells and WI-26 cells that is similar to that observed in primary MKC cultures. Studies are being conducted to characterize further and identify these agents.

Thus, in spite of the negative findings in the past in search for microorganisms, especially viruses, as the causative agents of chronic neurological diseases, the possibility of infectious etiology must now be re-evaluated in the light of newer concepts of latency, masking, and "slow growing" viral activity. Viruses known to cause chronic degenerative diseases of the central nervous system in animals, such as scrapie and visna of sheep, have not, as yet, been clearly implicated in any disease in humans, though neutralizing substances to visna virus have been reported in human sera (11),

TABLE IV.—Attempts To Isolate a Transmissible Agent From Amyotrophic Lateral Sclerosis (ALS) and Parkinsonism Dementia (PD)¹ Occurring on Guam

Patient identification	Specimen for inoculation	Method of preservation	Experimental host range by date of inoculation and culture				
			Chimpanzee	M.rhesus	Primates M.cynomolgus	C.aethiops	Other mammals or avians
ALS:							
Naganta	CNS	CO ₂		September 1963	September 1963	September 1963 January 1964	February 1964
Taitingfong	CNS	CO ₂			September 1963 January 1964	September 1963	February 1964
Santos	CNS	CO ₂			September 1963 January 1964	September 1963 January 1964	February 1964
Villagomez ²	CNS	N ₂		July 1964			
PD:							
Artero ²	CNS	N ₂		July 1964			
Borja ²	CNS	N ₂		July 1964			
Gogue ²	CNS	N ₂		July 1964			
Domingo	CNS	CO ₂			September 1963 January 1964	September 1963	February 1964
Toves	CNS	CO ₂	August 1963		August 1963 January 1964	August 1963 January 1964	February 1964

¹ Parkinsonism dementia syndrome on Guam associated with amyotrophic lateral sclerosis. ² Tissues from these patients pooled, homogenized, and inoculated in to animals.

TABLE V.—Attempts To Establish in Primates the Zil'ber Agent From Second Passage Monkey Brain (U.S.S.R.)

Inocula	Concentration	Host species	Dose/Route	Date of inoculation
Monkey PEAN N3098, brain + cord	10 ⁻¹	M. rhesus	0.2 ml./i.c.	Aug. 26, 1964
PEAN N3098, Seitz E/K filtrate	10 ⁻¹	M. rhesus	0.2 ml./i.c.	Aug. 26, 1964
PEAN N3098, Millipore 220 mμ filtrate	10 ⁻¹	M. rhesus	0.2 ml./i.c. ¹	Aug. 26, 1964
Serial dilutions of PEAN N3098 CNS and undiluted serum from human ALS patients in U.S.A. ²	10 ⁻¹	M. rhesus	0.2 ml./i.c.	Aug. 26, 1964
	10 ⁻³	M. rhesus	0.2 ml./i.c.	Aug. 26, 1964

¹ Animal died Nov. 23, 1964 with bloody diarrhea and dehydration due to heavy bacterial infection.

² Serum-virus mixtures incubated at room temperature for 2 hours before inoculation into animals.

NOTE.—Second passage monkey CNS tissue received from Dr. L. A. Zil'ber (6).

and in at least two instances reports have been made suggesting scrapie-like illness in sheep inoculated with tissues of the central nervous system from patients with subacute encephalitis (8) and acute disseminated sclerosis (10). Shubladze et al. isolated a virus from a case of acute encephalitis which they described was neutralized by a number of serums obtained from patients with disseminating encephalomyelitis or multiple sclerosis (12), but which was subsequently identified by Dick et al. to be a strain of rabies virus (13). Chumakov has reported the isolation on one occasion of Russian spring-summer encephalitis virus from the brain of a patient suffering from Kozhevnikov's epilepsy (28), a chronic convulsive syndrome which has often been suggested to be a late effect of this virus infection (2). The Russian workers have also suggested the possibility that Vilyuisk encephalitis, a chronic degenerative central nervous system disorder of Yakuts people in Siberia, is of viral etiology (4, 29, 30, 31, 32). In none of these cases is a viral etiology established, however. In fact, the relationship of the known scrapie, visna, and Aleutian mink disease viruses to any of the chronic degenerative nervous system dis-

eases of man, as well as the relationship of these human diseases one to another, as, for example, between amyotrophic lateral sclerosis and parkinsonism-dementia on Guam, and between these syndromes and similar syndromes in America and Europe, cannot be established, even if a transmissible virus is isolated from them, without a specific diagnostic test for the agents. Thus, we are also at present working on the production of hyperimmune animal sera to various particulate and macromolecular tissue fractions of human brain tissue from the diseases under investigation, as well as of tissue from scrapie-infected animals. These antisera may eventually serve as disease specific reagents in neutralization, complement fixation, haemagglutination-inhibition, fluorescent antibody and other serological tests which will be essential in order to establish a micro-organism as a cause of any of these illnesses.

Finally, it is significant to note that we have been able to culture in vitro for long periods of time viable cells from surgically biopsied brain tissue from a patient with ALS and more recently from the brain tissue of a patient with multiple sclerosis; we have also been able to recover viable cells in vitro from CNS

TABLE VI.—Attempts To Isolate in Primates a Transmissible Agent From Other Chronic Diseases Affecting Man and Animals

Syndrome	Tissue for inoculation	Number of cases	Duration of experiments (in months to date) ¹	Number of animals on test	Uninoculated controls
Dawson's inclusion encephalitis	CNS biopsy	1	11	2	1
Myasthenia Gravis	Thymus ²	1	11	2	1
Suspect Aleutian mink disease in man.	Biopsy lymph node	1	8	2	2
	Biopsy liver kidney pool		8	2	2
	Urine		8	2	2
Scrapie (Compton goat strain)	Mouse brain 4th passage ³	1	11	4	1

¹ As of July 1965.

² Viable cells from thymus gland obtained at surgical biopsy have been cultivated and subcultured in vitro continuously since Aug. 19, 1964.

³ Viable cells from scrapie infected mouse brain tissue have been cultivated in vitro.

tissues from kuru patients following preservation of the tissue up to 6 months in liquid nitrogen. In addition, in collaboration with Mrs. Hope Hopps, Division of Biologics Standards, National Institutes of Health, we have successfully cultured and serially subcultured viable cells of the thymus gland from a patient with myasthenia gravis. Supernatant fluids from these cultures, rich in cellular products, are being injected into animals and other cell lines in attempts to isolate an etiological agent. These fluids are being tested also for antigenic activity against a wide variety of standardized known specific viral antisera in an effort to establish a relationship to known agents.

ADDENDUM

During the several months subsequent to the presentation of this paper at the workshop-symposium we have further expanded our program to include inoculation of primates and a variety of small laboratory animals and tissue culture lines with CNS tissue specimens obtained at surgical biopsy and early autopsy from additional types of subacute and chronic diseases of the CNS of humans. The types of cases, the method of collection, and the number of months inoculated primates have been under observation are illustrated in table VII. In general, the results, after only 3 to 5 months' observation, are negative for clinically recog-

TABLE VII.—Summary of Additional Isolation Attempts

Case No.	Diagnosis	Tissue	Source	Number of months after inoculation into experimental hosts ¹
002696	Multiple sclerosis	CNS	Surgical biopsy	5
002706	Multifocal leuco-encephalopathy	CNS	Autopsy	4
002707	Necrotizing encephalitis	CNS	Autopsy	4
002708	Amyotrophic lateral sclerosis (sporadic)	CNS	Autopsy	3
002709	Parkinsonism dementia	CNS	Autopsy	3
002710	Schilder's disease	CNS	Autopsy	3
002711	Multiple sclerosis	CNS	Autopsy	4
002724	Multiple sclerosis	CNS	Autopsy	4
002766	Multiple sclerosis	CNS	Surgical biopsy	3
002767	Parkinsonism	CNS	Surgical biopsy	3

¹ As of August 1965.

nizable neurological disease. On the other hand, in one instance (case 002707) described as acute necrotizing encephalitis we were able to isolate a strain of herpes virus on rabbit cornea and in tissue cultures inoculated with a suspension of CNS tissue from the patient. This isolation confirmed the earlier isolation by Dr. J. Craighead, Peter Bent Brigham Hospital, Boston, Mass., of herpes virus from the same tissue specimens (33).

Finally, although several of the inoculated primates died of acute infection during the period of observation, as noted in our paper, none had developed signs suggestive of chronic neurological disease until the recent onset in two female chimpanzees. The first of these, inoculated 20 months previously with a suspension of frozen brain material from a kuru patient, has developed progressive incapacitating cerebellar signs with ataxia and tremors; the second, similarly inoculated with a suspension of brain material from another kuru patient, has developed, 21 months after inoculation, slight wasting lassitude and some tremor which appears to be progressive. Whether these syndromes are spontaneous or related to the inoculation remains to be determined.

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DISCUSSION

APPEL: I would like to make a comment with regard to the tissue culture aspect of your findings. Most of what has been described here are diseases that are going to afflict neurons; one of the things that we found is that it is extremely difficult to maintain neurons when you take tissue from newborn animals and transplant it into culture as explants. Even with suspension cultures, once you get into the problem of tissues from adult patients, or from a variety of different mammalian species, you are in great difficulty in trying to sustain the neuron—let alone having frozen it in liquid nitrogen, or having taken it at various periods after the death of the individual. I would suspect that you have no neurons at all in these cultures but only fibroblasts.

GIBBS: I would certainly agree with you that we are dealing with diseases that affect neurons, and that the "crudeness" of some of our techniques would probably not maintain neuronal growth, let alone assure viable neuronal recovery following storage in liquid nitrogen. However, the significance of our tissue culture studies must be considered in the light of our speculations about the viral etiology of the neurological diseases being discussed at this workshop, indeed we might include all progressive degenerative diseases of the nervous system, and by culturing viable cells from liquid nitrogen-preserved CNS tissue and from freshly obtained surgically biopsied CNS tissue we are able to create a

controlled environment in which to study them. Now, it is quite true that the cells we are culturing may not be those which the pathologists describe as being most affected, but there is no evidence thus far to show that these cells do not or cannot be supporting virus growth and maturation, in an endosymbiotic state as recently described by Dr. Koprowski. And even if this were not directly observable, based on CPE in cell cultures or disease in an experimental host, we are still in the excellent position of being able to study these newly established cell lines for clues towards the establishment of an infectious etiology by testing the supernatant fluids and various components of cell fractionation for antigenic profiles.

GAJDUSEK: I agree with Dr. Gibbs' explanation. We are interested in these cell cultures whether the cells are neurons or not. They come from the patient and we would have no a priori knowledge whether a virus producing neuronal death was not also growing in cells other than neurons.

KOPROWSKI: Based on your previous observation it is not unlikely to expect that the virus growing in the central nervous system may grow as well in fibroblasts or in epithelial cells.

APPEL: However, if it is negative, you have not disturbed the possibility that an agent may still be flourishing in neurons.

JOHNSON: In relation to both your work and Bunina's I would like to bring up the point that diseases caused by a virus may differ from host to host, as exemplified by Coxsackie viruses, etc. Bunina in Zil'ber's work claims to be reproducing amyotrophic lateral sclerosis in monkeys; according to Hirano's pathology they have not reproduced ALS in monkeys. On the other hand, they do have something they are transmitting, which is causing muscle weakness.

Unfortunately, they have not looked at the muscles; they have not looked at peripheral nerves. It can well be that either the virus of ALS, if such exists, causes peripheral neuropathies or myositis in monkeys. Relative to this same point I was very curious about your nonspecific deaths. Are those being looked at in detail?

GIBBS: Yes, all experimental animals which have died post inoculation, regardless of the interval, are undergoing isolation studies in our laboratory and complete neurohistological study in the laboratory of Mrs. Beck and Professor Daniel in England. In most instances, however, primate deaths have been directly attributable to severe parasitism or diarrhea due to salmonella or shigella, associated with rapid and severe dehydration; further, our largest losses occurred in a poorly "preconditioned" group of monkeys recently received from the jungle.

BURGER: With respect to the case of suspected Aleutian mink disease in man, was any of that material inoculated into mink?

GIBBS: We have not inoculated it into mink; we do not have a mink colony here at the N.I.H.

KENYON: Is this from the patient R.T.?

GIBBS: Yes. The material was received through the courtesy of Dr. Dessel of the Veterans Hospital in Wisconsin.

KENYON: We have inoculated 10 mink with urine from the patient R.T. We filtered the urine through a 220 m μ millipore filter and then inoculated 1.0 ml. intraperitoneally into pastel mink which were heterozygous for the Aleutian gene: 2 of the 10 developed Aleutian disease. None of 10 uninoculated controls developed Aleutian disease. However, since the number of animals affected was so small, one must be cautious in interpreting this data.

Transmission Experiments with Multiple Sclerosis

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INTRODUCTION

The association of multiple sclerosis (MS) with rural or agricultural pursuits suggested by some early epidemiologists has not been borne out by later studies. However, it did receive an important impetus from the report by Campbell et al. (1) that MS had occurred in four of seven research workers studying swayback at Cambridge, England. In 1951 Sutherland and Wilson (2) published an account of some attempts they had made to infect sheep by injection of CSF and blood from early cases of MS. These results were rather indefinite and they concluded: "This work has afforded no evidence in support of the suggested relationship between disseminated sclerosis and either swayback or scrapie in sheep." Their experiments embraced the period November 1948-May 1950, i.e., some 17 months.

However, studies initiated by the late Dr. Sigurdsson at the Keldur Institute, Reykjavík, and extending over the years 1948-56 suggested that certain virus diseases were of exceptionally long latent or incubation period. Stimulated by studies of visna (which was at first thought to be a demyelinating disease) groups of sheep were inoculated intracerebrally with brain material from acute cases of MS, but no visna-like condition developed over the 6 to 12 months the animals were observed.

MATERIAL AND METHODS

At the end of September 1959 there became available in Newcastle the brain of a patient (SELL) in her early 40's who had died of an acute MS attack, which had virtually transected her medulla. Certain unusual features of this case (together with clinical history) were the subject of a report by Field, Miller, and Russell (3).

A. Experiments in Newcastle

Experiment I (September 1959).—The brain (SELL) was obtained within 4 hours of death and immediate inoculations made by one of us (E.J.F.) into a variety of experimental animals, including four sheep. These animals were the offspring of a cross-bred ewe (Scotch Blackface × Hexham Leicester) and a Clun ram, and were of a type believed not to be susceptible to natural scrapie (because at the time of these experiments it was thought that a visna-like condition might emerge from the experiments). 0.5 ml of 1 in 10 suspension of material from the neighbourhood of a recent lesion in the frontal pole of the brain was injected in each case. Four control sheep were inoculated with normal human brain suspension.

Experiment II (July 1962).—Fourteen Cheviot lambs were inoculated soon after weaning with a freshly prepared suspension of SELL brain that had been held

for 22 months at -18°C . At the same time, 12 control animals were inoculated with normal human brain.

In both experiments all animals were allowed to run together on a common pasture.

B. Experiments in Reykjavík

Experiment I (April 1960).—Material from the same (SELL) brain was sent to Iceland where four sheep were inoculated and a like number of controls with normal human brain from Newcastle. 0.4 ml of a 1:4 lightly centrifuged suspension was inoculated intracerebrally.

Experiment II (February 1962).—Some of the original material used for inoculation in experiment I and which had now been in the deep freeze at -18°C . for about 29 months altogether was inoculated into five further sheep, using 0.4 ml. of a 1:4 suspension. One animal developed a scrapie-like condition after 19 months and another after 26 months. The remaining three animals have remained well.

Experiment III (February 1962), was a passage experiment. Material from sheep Nos. 987 and 989 of experiment I was inoculated in the usual way into five animals.

Experiment IV (March 1963).—Another sample of SELL brain which had been held for 30 months at -18°C . was sent out to Reykjavík and 0.3 ml. of 1:4 suspension injected into each of five sheep.

C. Experiments at Compton (October 1962)

Twenty sheep of the Herdwick breed (about 50 percent of which were known to be susceptible to scrapie) were inoculated intracerebrally with 1.0 ml. of 1 in 10 freshly made up suspension of SELL brain that had been held at -18°C . for 36 months. Twenty control animals were similarly inoculated with normal human brain. Six dairy goats were injected intracerebrally with the same volume of SELL brain and six with normal human brain as controls.

All animals were kept indoors in strict isolation in a building that had never housed scrapie-affected animals.

RESULTS

A. Newcastle

Experiment I (September 1959).—The sheep in this experiment were observed for 17 months. Cisternal puncture at intervals revealed no asymptomatic meningitis and when the experiment was terminated no pathological changes were found. The four control animals likewise were normal.

Experiment II (July 1962).—The 14 Cheviot sheep have not shown illness, and the 12 controls have likewise remained well.

Mice inoculated in September 1963 were observed for 14 months. Some appeared vaguely ill (rather hunched attitude with erected hair) but showed no clear-cut pathological changes under the microscope. Passage experiments from these animals are in progress.

B. Reykjavík

Experiment I (April 1960).—All of the original four sheep inoculated with SELL brain in Reykjavík developed neurological illness with the histological signs of scrapie (figure 1). Some details are set out in table I.

TABLE I

Sheep No.	Inoculated I.C.	Onset of symptoms	Incubation
986	8th April 1960	November 1961	19 months
987	8th April 1960	October 1961	18 months
988	8th April 1960	January 1962	21 months
989	8th April 1960	August 1961	16 months

The earliest signs of illness in these animals was a stiffness and clumsiness of gait affecting the hind limbs more than the fore. There was general nervousness and excitement and sometimes the ears were held in unnatural positions. Tremor made its appearance and was accompanied by ataxia. Neither teeth grinding nor scratching were observed. Nystagmus was seen in two animals in late stages of the disease. Whilst the early picture did not resemble rida the later stages came to do so. None of the four control animals showed any disturbance.

Experiment II (February, 1962: 29 months old SELL material).—Two of the five sheep inoculated developed signs, one at 19 months and the other at 26 months. The remaining three have remained well.

Experiment III (February 1962: Passage experiment).—

Sheep No.	Incubation
1110	10 months
1111	14 months
1112	10 months
1113	11 months
1114	9 months

The signs and pathology were similar to those of experiment I but the shortening of the incubation period from an average of 18.5 months to 10.8 months

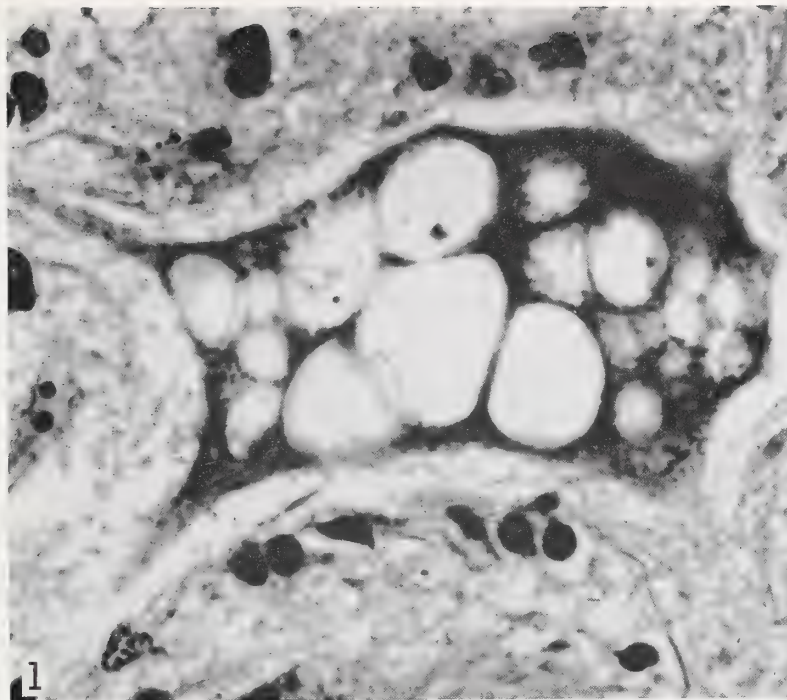


FIGURE 1.—Anterior horn cell of spinal cord (sheep No. 989) showing typical multiloculation of scrapie. (H.E. $\times 800$.)

was striking as also was the remarkable 100 percent take in these passage animals as with the originals in experiment I.

Experiment IV (March 1963).—One of the five animals developed the clinical and pathological signs of scrapie (fig. 2) after 15 months. At the time of writing, it is still under 2 years from inoculation and further cases may be expected. The one animal which did develop scrapie was also found to have a small localized cord softening in the lumbar region apparently from trauma or infection from repeated lumbar punctures. The lesion did not extend upwards and the brain was quite free from septic infection.

C. Compton

Twenty sheep inoculated in October 1962 have remained clinically well as have 20 control animals.

Of six dairy goats inoculated intracerebrally (I.H.P.) two have developed postural and behavioural abnormalities that were first noticed after 21 and 22 months respectively. Figure 3 shows the characteristic stance of the first of these animals, backed into a corner with the head down and slightly twisted, the ears incoordinated and the hind legs pulled slightly forwards. Compare the erect stance and alert appearance of the normal animal. The affected animal had periods of hyperactivity when it would chase its com-

panions, kick its hind legs in the air, leap at the wall and perform various other bizarre movements, and then, quite suddenly, it would back into the corner of the loosebox and remain there for long periods in a state of drowsy indifference to its surroundings. After 3 months' observation this animal was killed for histological examination of the C.N.S. and for subinoculation of tissues into other goats; at that time it was in good bodily condition and there was no evidence that the disease might prove fatal. No histological abnormalities were detected in the brain or spinal cord.

The second affected goat is still under observation. The abnormalities it shows are qualitatively similar to those in the first animal, and it becomes extremely disturbed if handled. The outstanding abnormality in this animal is restless pacing that may continue for hours.

The slow evolution of the condition in both animals appeared unlike scrapie.

The platelet adhesion test with encephalitogenic factor (EF) described by Caspary (4); Field and Caspary (5), was applied to blood taken on November 23d, 24th, and 25th, 1964 (i.e., 25 months after inoculation), from goats inoculated with SELL or normal brain. Results are shown in table II. Student's *t* test shows that "MS-goats" differed from "non-MS" animals ($P < .01$).

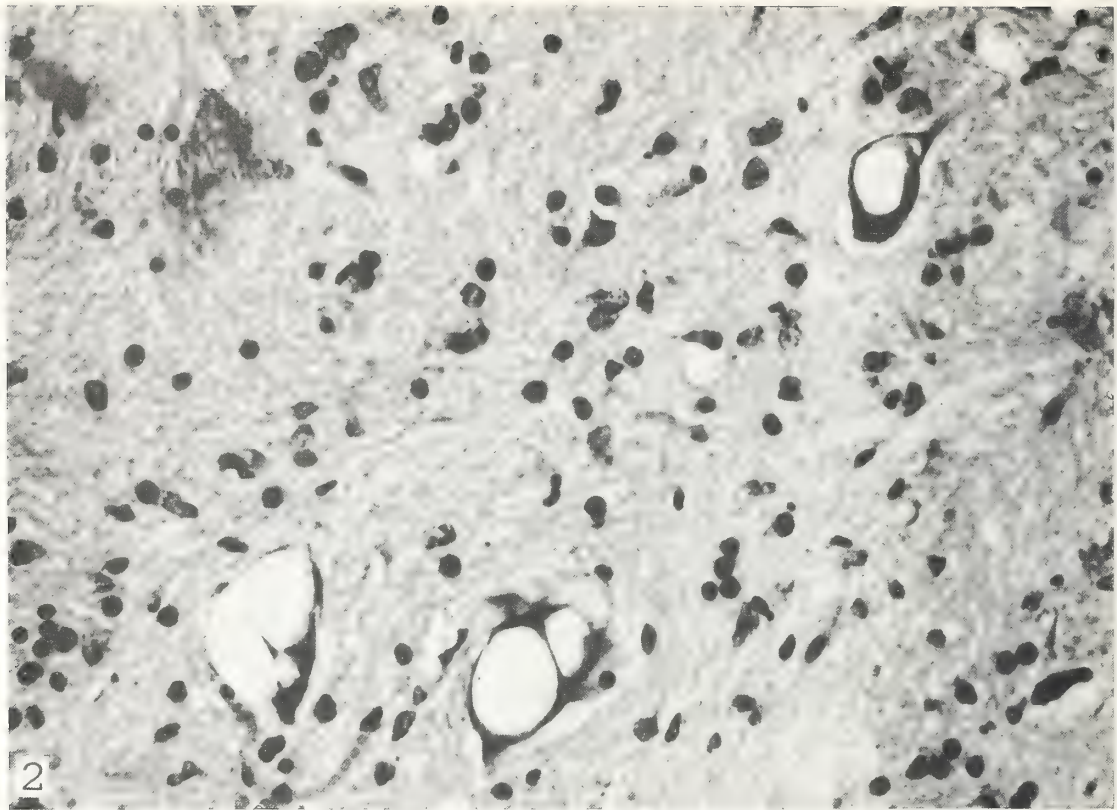


FIGURE 2.—Vacuolated cells in thalamic region of sheep with clinical signs of scrapie. (H.E. $\times 340$.)

TABLE II.—Platelet Adhesion Test With Human E.F.

"MS" Goats	Δ	"Non-MS" Goats	Δ
G 338	-10	G 291 Inoc. normal brain	0
G 328	-10	G 227 Inoc. normal brain	-1
G 208	-3	G 318 Inoc. normal brain	+6
G 265	-12	H 20 Clinical scrapie	0
G 327	-5	H 76 Not inoculated	-3

Student's *t* test $p < .01$.

DISCUSSION

It must be clearly stated at the outset that this paper is in the nature of a preliminary report which would have been held over but for the occasion of this workshop symposium. The main facts presented are:

1. Sheep inoculated with different samples of SELL brain in Reykjavik have on two occasions shown a scrapie-like condition and this has been passaged.
2. No sheep in England has done likewise.
3. Goats at Compton have developed an indefinite but real behavioural change unaccompanied by histological evidence of scrapie in the one animal so far killed.

Moreover, the evolution of the clinical disturbance has been slow and different from goat scrapie, though certain resemblances have been noted, e.g. the "anxi-

ous" expression and hypersensitivity. Further, some of these goats have shown a positive platelet adhesion test with human encephalitogenic factor, though one scrapie goat was negative.

In the passage experiment with the Icelandic sheep, the average incubation period diminished from the original 18.5 months to 10.8 months, suggesting the establishment of a new condition in the animals rather than the emergence of an already present latent condition. It should be emphasized at this point that the flock from which the animals were drawn is one in which rida (the Icelandic form of scrapie) has never been observed and that no rida experiments had been carried out in the particular cubicles in which the animals were housed though two rida transmission experiments were going on elsewhere in isolation units. We feel that "accident" can reasonably be rejected as an explanation of the Icelandic observations especially since four out of four animals were affected in the first experiment—a phenomenon which would be excessively rare had known scrapie agent itself been inoculated.

The 100-percent "take" in the passage experiment (with substantially shortened incubation period) also



FIGURE 3.—Affected goat inoculated with MS material (fawn) together with another animal (black) similarly injected but not showing signs. Note characteristic stance of affected animal—backed into corner, lowered and slightly twisted head, incoordinated ears and hind legs pulled slightly forward.

suggests that something new was being introduced and we were not simply dealing with the appearance of an already latent rida.

Moreover, the appearance of the scrapie condition in experiment IV where entirely new SELL material was used, again supports the genuineness of the findings.

The failure of sheep experiments in England calls for comment. Whilst the first Newcastle experiment was performed with animals which were not especially susceptible to scrapie (chosen at the time because one of us (E.J.F.) was then working on the supposition that visna might emerge) the Cheviot breed used in experiment II are about 30 to 50 percent susceptible. These have now been under observation for $2\frac{1}{2}$ years. Moreover, Herdwick sheep at Compton have shown no changes after 2 years. The wide variation in susceptibility to scrapie of different breeds of sheep is well established (Gordon (6)) and we hope to import a group of Icelandic sheep into England to carry out a further experiment on them at Compton.

The absence of clear pathological changes in the experimental goat killed is disappointing. However, something unusual is certainly amiss in these animals and the aberrant results obtained, both from the clinical and pathological points of view, when attempts are made to cross the species barrier are well known and have been emphasized elsewhere in this workshop symposium by one of us (I.H.P.). We await, therefore, with great interest the results of the passage experiments made from the one goat so far killed. The others will be observed for much longer periods.

The increased platelet adhesiveness to glass induced by EF in the "MS goats" and not in a known scrapie animal requires further comments. No extensive study of platelet adhesiveness in scrapie has yet been made and it may be a phenomenon which is limited to a certain phase of the disease. In the absence of more data, speculation would be fruitless but we plan to examine the sheep under experiment by the same test.

Finally, the present report may be regarded as an interim one giving strong encouragement for independ-

ent repetition of our experiments as suitable material comes to hand. Should it be confirmed that multiple sclerosis and a scrapie-like condition in sheep are linked, then many interesting speculations would be warranted.

SUMMARY

Intracerebral inoculation of brain material from an acute case of multiple sclerosis into sheep has on two separate occasions resulted in the emergence of a scrapie-like condition. Reasons are given for not regarding this as adventitious.

Goats similarly treated have shown an ill defined disturbance of behaviour and posture without pathological changes (in one animal so far sacrificed). They do, however, show increased platelet adhesiveness to glass under the influence of a human encephalitogenic factor.

Mice inoculated with the same material have not, to date, shown any pathological disturbance.

The authors are much indebted to Mr. E. A. Caspary for carrying out the platelet adhesion tests.

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DISCUSSION

HADLOW: Has all this work been done with specimens from the one case of multiple sclerosis?

FIELD: Yes. This has been done only with this very acute case of multiple sclerosis. In all Dr. Henry Miller's many years' clinical experience of multiple sclerosis, he has never seen a case which died of such an acute lesion. It was just as if she had had her medulla transected by a plaque.

GIBBS: I believe that you also put the inocula into mice. Did these mice show anything?

FIELD: Yes, they did show something. They looked in poor condition, hunched up with hair standing on end, and I actually made a film showing them walking about a bit. Sometimes I thought they had scrapie, sometimes it did not look like scrapie. I killed them and there was nothing found pathologically.

GIBBS: Did you pass them?

FIELD: Yes, I have passaged them but nothing has occurred so far.

HOTCHIN: How long did it take for them to show that condition?

FIELD: Something well over a year, and controls done at the same time have been perfectly normal.

ALPERS: This patient that the material came from had she had any connection with sheep?

FIELD: No, she did not have. She was a very peculiar case; she came into hospital in 1956 before I was in Newcastle, and she was diagnosed as "viral encephalitis," but no viral studies were made. About 18 months afterward, she began to develop signs of multiple sclerosis and, after a period of about 3 years or so, she went down with a final terminal episode with this very acute plaque. There is no question that histopathologically she was multiple sclerosis. She is, in fact, the patient in whom we found peculiar rodlike structures in vacuoles within glial cells. I might add that I do not believe these rods to be the causal organisms of multiple sclerosis.

HADLOW: When I was in Iceland I discussed these experiments with Dr. Pálsson. I came away with the story that in a particular area, or around particular farms, farmers followed what I gathered was an unusual practice of having the sheep intimately associated with a few cattle. It occurred to me that perhaps the virus was carried around by these cattle and when sheep were reintroduced and allowed to associate intimately with the cattle that this might have been the source of reinfection.

PATTISON: I must emphasize that the greatest possible care was taken in Iceland to keep the sheep free of rida, that they are not actually doing experimental inoculation with rida, and that the appropriate controls were negative. I think the possibility of some contamination with rida in these particular experiments is remote in the extreme.

FIELD: Also the original sheep that came down have been passaged, with a shortening of the incubation period, which suggests that this was something new that had been introduced. It was not the local strain of rida.

Epidemiological Evidence for a Viral Etiology for Multiple Sclerosis

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An attempt will be made to summarize here the results of a series of epidemiological studies of multiple sclerosis conducted by one group of investigators, both in the counties of Northumberland and Durham, England, and in Boston, Mass., and indicate the evolution of thought which has resulted in the hypothesis that multiple sclerosis may be an occasional manifestation of a widespread subclinical infection with a long incubation period, possibly of about 21 years.

Familial and Conjugal Studies

Initially, a study was undertaken of the familial occurrence of the disease. Analysis of a series of 700 consecutive patients with multiple sclerosis (1) referred by general practitioners indicated that in 4 percent of families 2 members had established multiple sclerosis. When families in which a second case of multiple sclerosis was strongly suspected but not definitely established were also considered, the familial occurrence was 4.9 percent. These figures are consistent with those reported by other investigators who have shown that the familial disease occurs 5 to 8 times as commonly as cases in the general population. Table I indicates the frequency of occurrence in relatives of known cases. Within affected families the disease is more likely to affect the female members than the male. An approximation of the prevalence of the disease in relatives can be made from these frequency data,¹ and it indicates that the disease is 7.8

TABLE I.—Frequency of Multiple Sclerosis in Relatives of Known Cases of Multiple Sclerosis in a Study of 607 Families¹

Affected relative	Cases in relatives	Exposed population	Frequency per 10,000 relatives
Female sibling	18	1, 079	164. 1
Male siblings	7	1, 072	65. 3
Mothers	5	603	82. 9
Fathers	2	603	33. 2
Total	32	3, 357	94. 5
Any sibling	25	2, 151	115. 0
Either parent	7	1, 206	58. 0
Any female in family	23	1, 682	135. 0
Any male in family	9	1, 675	53. 7

¹ Schapira, K., Poskanzer, D. C., and Miller, H.(7).

times more prevalent in the immediate relatives of patients than in the general population.

Examination of the data failed to reveal any readily recognizable genetic pattern and evidence collected elsewhere including twin studies (2) has thrown considerable doubt upon a purely hereditary explanation of the familial incidence. An increased incidence of multiple sclerosis in families has led to the common assumption that a genetic factor must play an important role in its etiology. Familial diseases are often confused with hereditary ones, however, even though instances of the same condition in two members of the same family may in fact reflect common exposure to a similar environmental factor rather than a genetically determined etiology.

Since the occurrence of familial cases cannot be explained by any simple genetic pattern, several other hypotheses might be entertained. The first is that the disease is in fact due to a genetic factor with incom-

¹ The prevalence of multiple sclerosis in relatives of patients can be approximated by multiplying the frequency in relatives (94.5/10,000) by the birth-rate in the mean years of birth of patients (1920-24; 213/10,000) by the mean duration of the disease (19.3 years). The result is 38.8/10,000 as compared with 5.0/10,000 in the general population.

plete penetrance, an explanation which can be manipulated to explain any disease with a familial occurrence. A second possibility is that there is "an hereditary predisposition" which produces overt disease only in the presence of an appropriate environmental stimulus. A third explanation postulates that familial multiple sclerosis is not due to a genetic factor at all but to exposure of members of the family to some common environmental factor.

Conjugal multiple sclerosis (cases in both husband and wife) occurred at the predicted rate in the general population. A low conjugal incidence of multiple sclerosis has been used as evidence against the hypothesis that an environmental factor is important, but it can equally be adduced to support the view that common exposure occurs early in life.

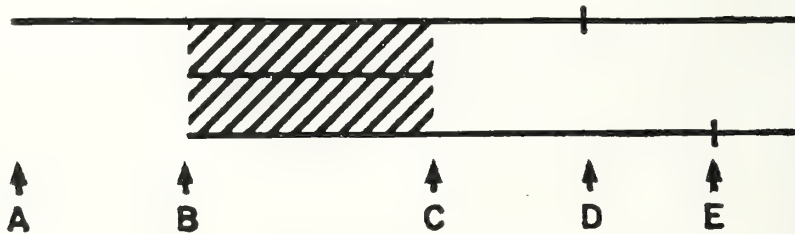
In view of the lack of evidence of a genetic basis for the fact that at least 4 percent of patients have affected siblings or parents, the possibility of exposure to a common environmental factor was hypothesized. It seems likely that if common exposure is responsible it probably occurs prior to marital age because conjugal multiple sclerosis occurs at the frequency expected in the general population.

An attempt is made in figure 1 to present in diagrammatic fashion the relationship of affected mem-

bers of the same family. Each person's life span is illustrated by a line indicating birth, onset of multiple sclerosis, and death. For any two affected members the hatched area indicates the period during which the two individuals lived together and might have been exposed to a common environmental factor. Figure 2 illustrates this relationship in 17 affected sib pairs.

Because the nature of a postulated environmental factor is unknown it is not clear whether a single exposure, multiple discrete exposures, or a continuous exposure over time might be required to cause the disease. A formula to calculate the "incubation period" was therefore developed, which weighted the period of common exposure for its length (fig. 1).

From these data it was possible to calculate for siblings an "incubation period" for multiple sclerosis of 21 years. The mean minimum incubation period was 12 years, the mean maximum incubation period 32 years. The mean age at onset in the siblings in this study was 34.9 years. If one accepts the assumption of the calculations set forth here, that the disease in siblings is due to common exposure in childhood, it may be tenuously concluded that common exposure to some exogenous factor may occur at about age 14, with a range of 3 to 23 years. Such calculations are



Calculation of incubation period from common exposure diagram

A—Year of birth of first case

B—Year of birth of second case and beginning of period of common exposure

C—End of period of common exposure

D—Onset of multiple sclerosis in first case

E—Onset of multiple sclerosis in second case

$\frac{BD + CD}{2}$ mean incubation period in first case

$\frac{BE + CE}{2}$ mean incubation period in second case

$\frac{BD + CD + BE + CE}{4}$ mean incubation period in each family

$\frac{\sum \left(\frac{BD + CD + BE + CE}{4} \right) BC}{\sum BC}$ mean incubation period in all families weighted by length of common exposure period in each family (assume each year of common exposure is a separate instance)

FIGURE 1

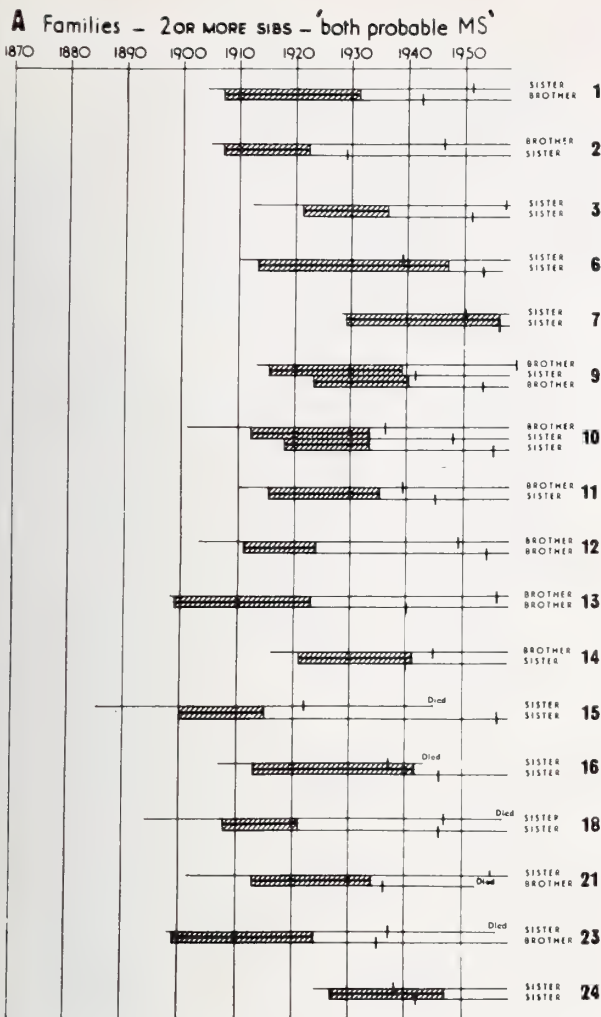


FIGURE 2.—Diagrammatic representation of periods of "common exposure" in 17 families with 2 or more siblings diagnosed as "probable multiple sclerosis."

at best arbitrary, but they attempt to focus in some numerical fashion on the possibility of common exposure in early life, possibly about the time of puberty, as a cause of multiple sclerosis. These calculations are presented in an attempt to evaluate the common exposure hypothesis of etiology in contrast to the genetic view. They also draw attention to the relatively early age at which exposure to an etiological factor in multiple sclerosis may occur, a possibility obscured by a too exclusive attention to events late in the life histories of affected patients.

Socio-Economic Studies

A trend toward higher socio-economic groups in cases of multiple sclerosis was first commented upon in the Registrar General's reports for 1930 and 1950

for certain age groups. Miller, Ridley, and Schapira, 1960 (3), comparing 659 patients with multiple sclerosis by socio-economic class (as indicated by the Registrar General's five classifications), suggested that the risk of a professional person in group I developing multiple sclerosis may be twice as great as that of an unskilled worker in group V in the same community. These data are uncorrected for the fact that a chronic disabling disease has a tendency to move patients downward in a socio-economic scale, and may therefore be an underestimate of the socio-economic distribution. However, Westlund and Kurland (4) believed that the various economic strata were about equally represented in patients and controls in 112 patients in Winnipeg, Canada.

Prevalence Studies

The prevalence study conducted in Northumberland and Durham (5) produced 1,156 cases for a prevalence rate of 50.1 per 100,000; 42.0 in males, 57.0 in females. The curve of age specific rates by age at onset, as illustrated in figure 3, is of particular interest. As pointed out by Acheson (6), the curve resembles that for a childhood infectious disease, a shape which

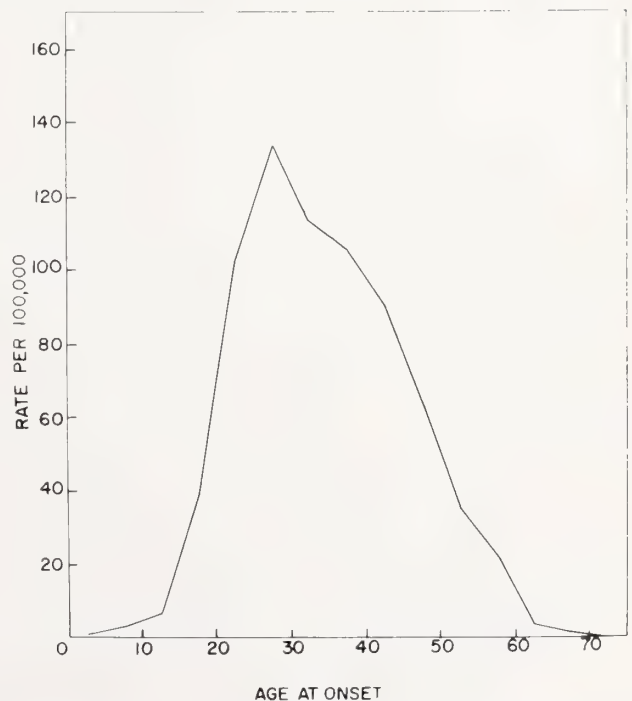


FIGURE 3.—Age specific rates at onset for multiple sclerosis (1156 cases in Northumberland and Durham, England, from Poskanzer, D.C., Schapira, K., and Miller, H. (5)).

rarely occurs for diseases with onset later in life. These data again suggest the possibility of a long incubation period.

No difference in prevalence rate was found between urban and rural areas, confirming results reported in other studies, or between the two counties analyzed separately. The age at onset, however, was significantly different at the one percent level when urban and rural areas were compared, with onset occurring later in the rural areas.

Large local fluctuations in the frequency of the disease in small populations have sometimes been reported. Our cases were assigned to 53 districts, and although they varied markedly from 19 per 100,000 to 85 per 100,000 compared to the overall rate of 50 per 100,000, these variations in community rates were not significantly different from the expected distribution for the occurrence of small numbers of cases in large populations (Poisson distribution), and no geographic pattern of higher prevalences was apparent. It appears that the local concentrations of cases reported elsewhere may reflect nothing more than chance distribution and variation in ascertainment of a rare disease in large populations.

Geographical Features

Certain of the worldwide geographical features of multiple sclerosis revealed by comparative prevalence and mortality studies require attention. It is generally conceded that the frequency of the disease increases with increasing latitude, probably in both hemispheres, and is rare in the tropics.

Studies of the prevalence of multiple sclerosis in various parts of the world suggest that multiple sclerosis is rare between the equator and latitude 30 to 35 degrees and becomes more common with increasing latitude. The disease has been shown to be associated inversely with total annual hours of sunshine and average daily solar radiation, but it appears to be unrelated to ultraviolet radiation, degree days (a measure of temperature), or adequacy of medical facilities (7).

The relationship of the disease to latitude is strikingly illustrated in figure 4, which is based on mortality data for multiple sclerosis in the British Commonwealth, plotted against latitude and compared with a similar plot of mortality data for amyotrophic lateral sclerosis (8). The careful studies of Kurland and his colleagues also illustrate this relationship, using similar

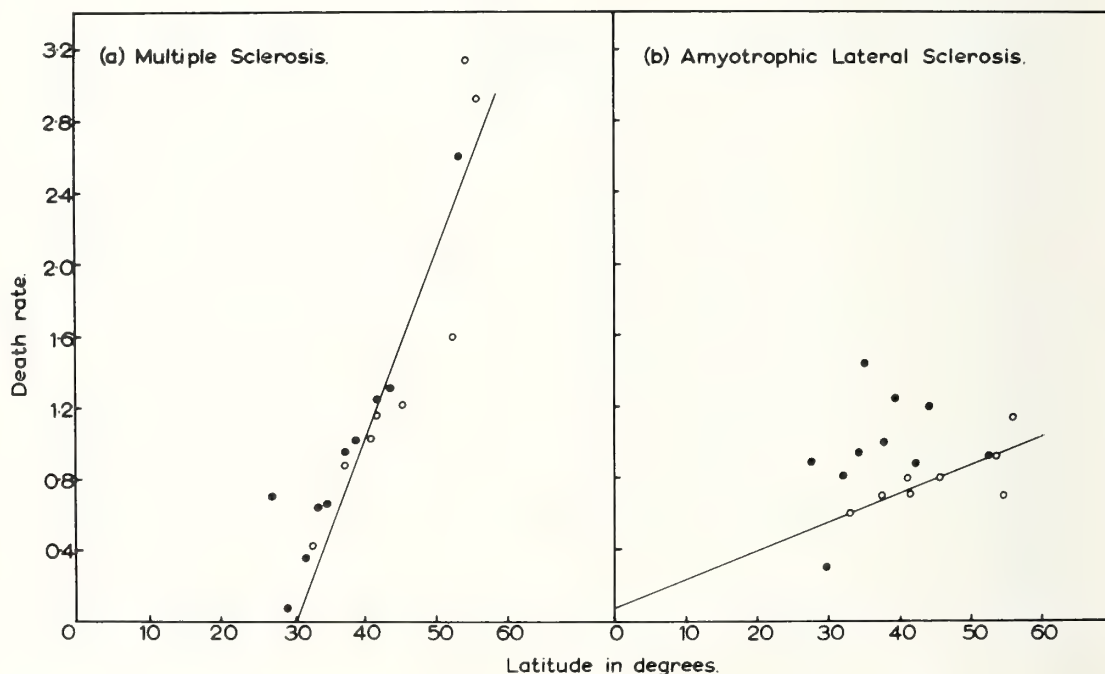


FIGURE 4.—Average annual age-adjusted death rates per 100,000 by mean latitude. (a) Multiple sclerosis; (b) amyotrophic lateral sclerosis. Black circles, Southern Hemisphere; open circles, Northern Hemisphere. From Acheson (8) reprinted by courtesy of the author and the British Journal of Preventive and Social Medicine.

diagnostic criteria in a series of North American communities as shown in table II (9). The exception to the pattern of geographical distribution appears in the study of the islands of Japan, where no differences in prevalence rates were found among four cities separated by ten or more degrees of latitude. In all of Japan the prevalence rate was low. The prevalence of multiple sclerosis in the Southern Hemisphere is not completely documented but also seems to be related to latitude, with the exception of unusually low rates in South Africa and Queensland.

TABLE II.—Multiple Sclerosis. Prevalence Ratio for White Population, United States and Canadian Communities (9)

Community	Latitude north	Prevalence rate per 100,000 population
Winnipeg	50	42 (40) ¹
Boston	42	41
Denver	40	38
San Francisco	37	30
New Orleans	30	13 (6) ¹

¹ Final result after detailed neurological examination of patients.

An immigrant from a higher risk area to a low risk zone appears to carry a high risk with him, as in South Africa where the disease is rare in native born white South Africans compared with European born white South Africans, and particularly in Israel where the prevalence in groups migrating to Israel correlates with the risk in their countries of origin (so that it increased with latitude), showing a tenfold difference between the highest and lowest prevalence rates by country of origin (10). The long delay in the onset of multiple sclerosis after migration to a low-risk area (up to 20 years in South Africa with a minimum latent period of about 13 years and a minimum latent period for immigrants to Israel of 9 years), suggests a long incubation period whether the disease was acquired prior to immigration or after arrival in the low-risk area.

The geographical distribution of multiple sclerosis in association with the calculated incubation period for the disease, indicating onset early in life, first suggested the analogy to the epidemiology of paralytic poliomyelitis (11). In poliomyelitis a similar variation occurs with latitude. It is postulated that in equatorial regions where sanitation is poor, infection with poliomyelitis virus is universal early in life when the risk of paralytic disease is less; cases later in childhood and in adult life are rare. Rising incidence of paralytic poliomyelitis is related to improvement in the level of sanitation. Paralytic disease is therefore more common

with increasing latitude because sanitation is generally superior in the temperate regions.

The risk of paralytic poliomyelitis increases with travel from a high-incidence area for paralytic disease, such as the United States, to regions of low incidence, such as equatorial countries. As noted by Paul, the disease was unexpectedly prevalent among Allied troops in China, India, Japan, the Middle East, and the Philippines (12).

It may be significant that Japan is the only area in which prevalence surveys have been conducted where human excrement is universally used as a fertilizer. As in poliomyelitis, this may be a source of early widespread infection with an agent related to the etiology of multiple sclerosis. The epidemiological pattern of poliomyelitis in Japan is that typical of warm areas of the world where sanitation is primitive (12).

In poliomyelitis no difference in prevalence rates is noted between urban and rural areas, but as illustrated in figure 5 onset occurs at a later age in rural areas than in the urban ones (13). For comparison, the difference between age at onset in urban and rural areas for multiple sclerosis in Northumberland and Durham is illustrated in figure 6 (11).

The proclivity of mortality and morbidity in poliomyelitis for the higher socio-economic classes as a result of its relationship to sanitation has been well demonstrated. The low rates of the occurrence of multiple sclerosis in Queensland and South Africa

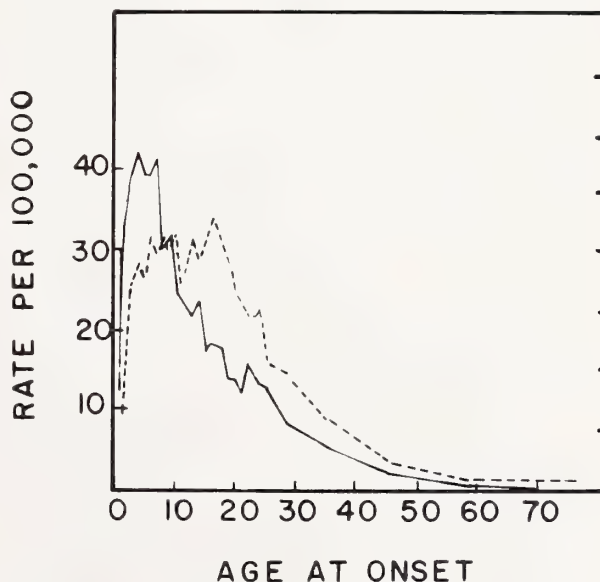


FIGURE 5.—Comparison of ages at onset of cases of paralytic poliomyelitis in Sweden by urban and rural distribution (age-specific rates). Solid line—cities and towns. Dotted line—rural communities.

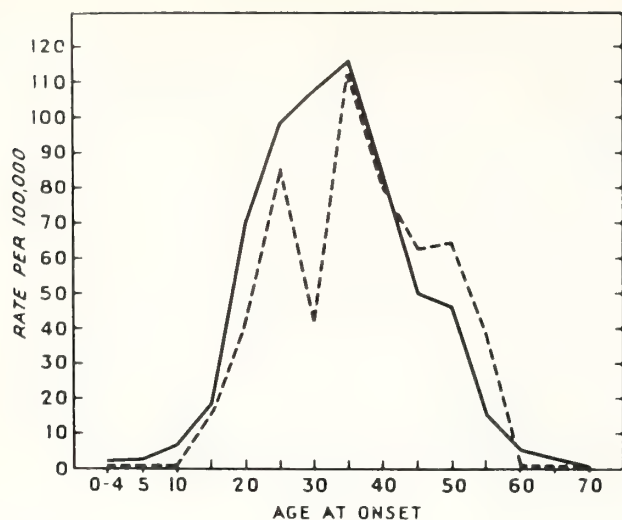


FIGURE 6.—Comparison of ages at onset of cases of multiple sclerosis in Northumberland and Durham counties by urban and rural distribution (age-specific rates). Solid line=case-rates per 100,00 in cities over 65,000 population. Dotted line=case-rates per 100,000 in rural districts.

where sanitation is excellent are inconsistent with the hypothesis of enteric spread infection, but in South Africa servants are universally available. It has been pointed out by Chambers (14) that when cholera occurred in the United States it was more prevalent in the higher socio-economic groups in the southern United States where the use of servants was more frequent.

The fact that more than 50 percent of South African-born patients with multiple sclerosis have spent some time in Europe, whereas among the South African-born population at risk only about 5 percent have visited Europe, may well be correlated with the fact that those visiting Europe are from higher socio-economic classes. The increased risk of multiple sclerosis for immigrants does not hold for Queensland, and the rate does not seem to be higher in British immigrants than in native-born Australians.

The familial occurrence of paralytic poliomyelitis, which in a typical epidemic was 4.3 percent for secondary cases of paralytic disease in a family already affected, is comparable to that of 4.9 percent in households with multiple sclerosis. The risk of the family as compared with the general population is 8.5 to 1 in paralytic poliomyelitis, 7.8 to 1 in multiple sclerosis (11). Even the incidences of the diseases in Northumberland and Durham seem to be similar, paralytic poliomyelitis having a lifetime incidence of 2.8 per 100,000 as compared with the incidence of multiple sclerosis of 2.6 per 100,000 found in our studies.

TABLE III.—Tonsillectomy in Patients With Multiple Sclerosis Compared With Their Siblings

	Tonsillectomy		No tonsillectomy		Total	
	Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent
Patients	133	63.3	77	36.7	210	100.0
Siblings	106	50.5	104	49.5	210	100.0
Total	239		181		420	

$$\chi^2=7.1, .01>p>.001.$$

The two diseases share a number of provoking factors. In both diseases an increased risk in pregnancy is recognized. In poliomyelitis risk of clinical infection is increased by 59 percent in pregnant women as compared with nonpregnant women of child-bearing age. The increased risk of an exacerbation in the pregnancy year in multiple sclerosis as compared with nonpregnancy years is 50 percent (15). Trauma and excessive exertion, which seem to increase the risk of paralysis and localization in poliomyelitis, may have a similar effect in multiple sclerosis (16).

Tonsillectomy in Multiple Sclerosis

As epidemiological data accumulated which suggested a long incubation period for multiple sclerosis, a case history study of childhood events was undertaken in Boston comparing patients with their nearest siblings and their spouses (17). As indicated in tables III and IV, the tonsillectomy rate was significantly increased at the .01 level in 210 patients compared with their sibs, and 190 patients compared with their spouses. The differences could not be accounted for by differences in age or sex of the groups compared.

In order to examine the adequacy of medical history in patients as compared with their healthy sibs and spouses, 122 were examined by an otolaryngologist without benefit of history. In all but 12 cases the history of tonsillectomy was confirmed by examina-

TABLE IV.—Tonsillectomy in Patients With Multiple Sclerosis Compared With Their Spouses

	Tonsillectomy		No tonsillectomy		Total	
	Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent
Patients	121	63.7	69	36.3	190	100.0
Spouses	94	49.5	96	50.5	190	100.0
Total	215		165		380	

$$\chi^2=7.8, .01>p>.001.$$

tion. When the examination results differed from the history, examination tended to confirm the increased rate of tonsillectomy in patients.

These data indicate that the relative risk of acquiring multiple sclerosis of persons who have had tonsillectomy is 1.7 to 1 as compared with siblings or spouses who have not had tonsillectomy. These results are remarkably similar to the increased risk of paralytic poliomyelitis in previously tonsillectomized populations, which is 1.7 to 1 as compared with non-tonsillectomized groups (18). No effect of prior tonsillectomy on the initial symptom in multiple sclerosis was found comparable to the considerable increase in risk of bulbar poliomyelitis following tonsillectomy. However, the initial symptom in multiple sclerosis is often difficult to ascertain, particularly after a period of years.

Certain epidemiological features of multiple sclerosis are different from those for poliomyelitis or similar enteric viral infections. No seasonal incidence has been demonstrated for multiple sclerosis, nor have epidemic years been detected, but both may be blurred by the long incubation period and difficulty in establishing time of onset.

Poliomyelitis in epidemic form has occurred in north-east England only since 1947, and if one assumes the 20-year incubation period it is possible that an increase in multiple sclerosis may also be expected later if it too is related to sanitation. All available studies from other places indicate that the disease is either stable or increasing in frequency but not decreasing.

SUMMARY

Analysis of the epidemiological data on multiple sclerosis, based largely on our own studies in Northumberland and Durham, indicates that the age of acquisition of multiple sclerosis may be as early as 14 years, with the onset of clinical illness only after a delay of many years. It also suggests certain striking similarities between the epidemiological patterns of multiple sclerosis and paralytic poliomyelitis. Similar features include a geographical variation in prevalence which increases with increasing latitude, a similarity in urban and rural attack rates with a higher age of onset in the rural population, a similar increase in risk of clinical disease for other members of the family as compared with the general population, a shift toward the upper social classes in distribution of the disease, and an increased risk following tonsillectomy and with pregnancy. The epidemiological features of multiple sclerosis are compatible with two hypotheses: (1) that multiple sclerosis has a long incubation period, possibly about 21 years; and (2) that multiple

sclerosis may be an occasional manifestation of a widespread subclinical enteric infection, just as paralytic poliomyelitis is a rare manifestation of infection with poliovirus. Alternatively, multiple sclerosis may be an unrecognized rare complication of a widespread common clinical illness of childhood.

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DISCUSSION

MARGOLIS: One of the most provocative features of the epidemiology of poliomyelitis is the effect of immunization during a period in which there is epidemic disease and in which we assume that there may be a viremia. After the immunization, and a suitable incubation period, we may observe poliomyelitis maximally in the part of the spinal cord where the immunization would presumably alter permeability of blood vessels. Have you followed this up in relation to multiple sclerosis?

POSKANZER: There is a series of cases in the literature now showing that in situations in which trauma has occurred, in general, multiple sclerosis has begun in that particular extremity. But we do not have any evidence with relation to inoculations.

MARGOLIS: The data on poliomyelitis suggest that there is a viremia and that the trauma makes vessels more permeable and, therefore, the virus gets into the nervous system. You assume, conversely, that the trauma allows viruses to get out of some residue in the brain?

POSKANZER: I am not sure that this is a virus of the central nervous system as seen in the maedi-visna situation. I am not sure that there isn't a systemic disease which corresponds to multiple sclerosis that we just have not recognized. In the relationship between streptococcal disease and rheumatic fever, it was fortunate that epidemics of streptococcal disease were followed 3 weeks later by epidemics of rheumatic fever. The temporal relationship was readily apparent. In multiple sclerosis we are really looking for a needle in a haystack, but it is not necessary to assume that this is a virus of the nervous system breaking out; it might be a systemic virus breaking in.

FIELD: Some years ago we tested the effects of trauma or stimulation of peripheral nerve on the blood flow in the corresponding segments of the spinal cord in the rabbit by means of thermocouples introduced into the cord at two levels, and were able to show that there was a differential increase in blood flow in the segments corresponding to the peripheral nerve stimulated. In about 5 out of 60 experiments,

however, instead of having a sudden increase in blood flow in that segment, there was a very marked spasm of the vessels which reduced the blood flow, and this persisted for about twenty minutes. Later this work was confirmed by other workers. So it seems established that when you have peripheral nerve stimulation or peripheral exercise there is dilatation of vessels and increased blood flow in the corresponding segments of the spinal cord, and this should facilitate entry of virus in from the blood. On rare occasions, you seem to get in some animals sudden constriction of vessels.

KURLAND: I should like to say that I think that Dr. Poskanzer has presented a plausible hypothesis—that multiple sclerosis may be the result of the childhood effect of some enteric infection. However, I would raise some question about some of the items which he has compared. These items are selected, as Dr. Poskanzer knows, and it should be pointed out that one could find data, and much of it well documented, which would show the opposite of what he has described. For example, the shift towards the upper classes in multiple sclerosis: studies by Westlund in Winnipeg comparing multiple sclerosis patients and a sample of the population of the same community failed to show any such selection by social class. There are, I admit, new data coming from studies in veterans with multiple sclerosis that show a distinct selection by social class; nevertheless, the item is still controversial. As far as the sex ratio is concerned, most of the recent studies indeed have shown higher female to male ratios; however, there are several series and community studies where incidence in males is equal to or exceeds that in females. Whether we are beginning to see some important shift in the sex ratio, I do not know. As far as pregnancy is concerned, there are several articles which I think argue equally well against an increased risk during pregnancy. As far as trauma is concerned, I think there is little good evidence of such an association. The relationship of immediate prior trauma could also argue against the thesis of a long incubation period which many of us are inclined to accept for multiple sclerosis. We appreciate that in neurological diseases, the presence of some weakness or difficulty prior to the recognized onset may predispose to trauma. An association with trauma is not convincing in any of the studies that I have seen. There was no indication that trauma was a significant factor in the comparative study which Westlund carried out in Winnipeg.

I think the same applies to excessive exertion. Now, as far as the age at onset differing in rural and urban areas, this is based on the one study by Poskanzer and his associates. Multiple sclerosis is a disease with an appreciable error of diagnosis in its early stages and one in which errors in recall can easily affect recorded age of onset. I would hesitate to make a definitive statement of differences in age of onset for urban and rural populations until more data were available. I would urge Dr. Poskanzer to substantiate with further studies the relationship to prior tonsillectomy that he noted because of the striking data he has presented.

Finally, we are aware that there are differences in the geographic distribution, and I agree that this disease, undoubtedly, has an important exogenous factor. Consequently, an enteric infection is as good a candidate as any and better than most in the etiology of multiple sclerosis.

POSKANZER: I do not really defend the relationship to trauma. There have been several papers in the literature lately listing groups of cases in which trauma has been associated with the onset of multiple sclerosis but, as in any situation where one merely accepts case reports, there is little scientific evidence. In the matter of pregnancy, I disagree a little bit more. There is not much question from our data about pregnancy that there is an increased risk in pregnancy in this disease, although there is some suggestion that the risk may be actually more in the three months after delivery than in the nine months of pregnancy. As a matter of fact, we have as many exacerbations and onsets in the three months following delivery as we have in the remainder of the pregnancy year. As I said before, the intention of this presentation was to be provocative. I would like to see if virological data can prove or disprove this contention.

Epidemiological Changes in Kuru, 1957 to 1963

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Dr. Gajdusek has outlined the role kuru has played in bringing together this workshop and summarized some of the investigations aimed at trying to obtain a transmissible agent from the disease (1). Many reports from him and Dr. Zigas, and other workers, have traced the evolving knowledge about kuru since the discovery of the disease by Western medicine in late 1956.

Clearly there are many aspects of kuru which still arouse considerable argument, but one thing seems undisputed—where it occurs (figs. 1, 2, 3). The disease is found nowhere but in the designated kuru region shown in these maps, except in cases where people from this region have temporarily migrated out and come down with the disease in their new environment.

KURU REGION and OTHER FOCI of NEUROLOGICAL DISEASE in NEW GUINEA



FIGURE 1

LINGUISTIC BOUNDARIES - EASTERN HIGHLANDS DISTRICT TERRITORY OF NEW GUINEA

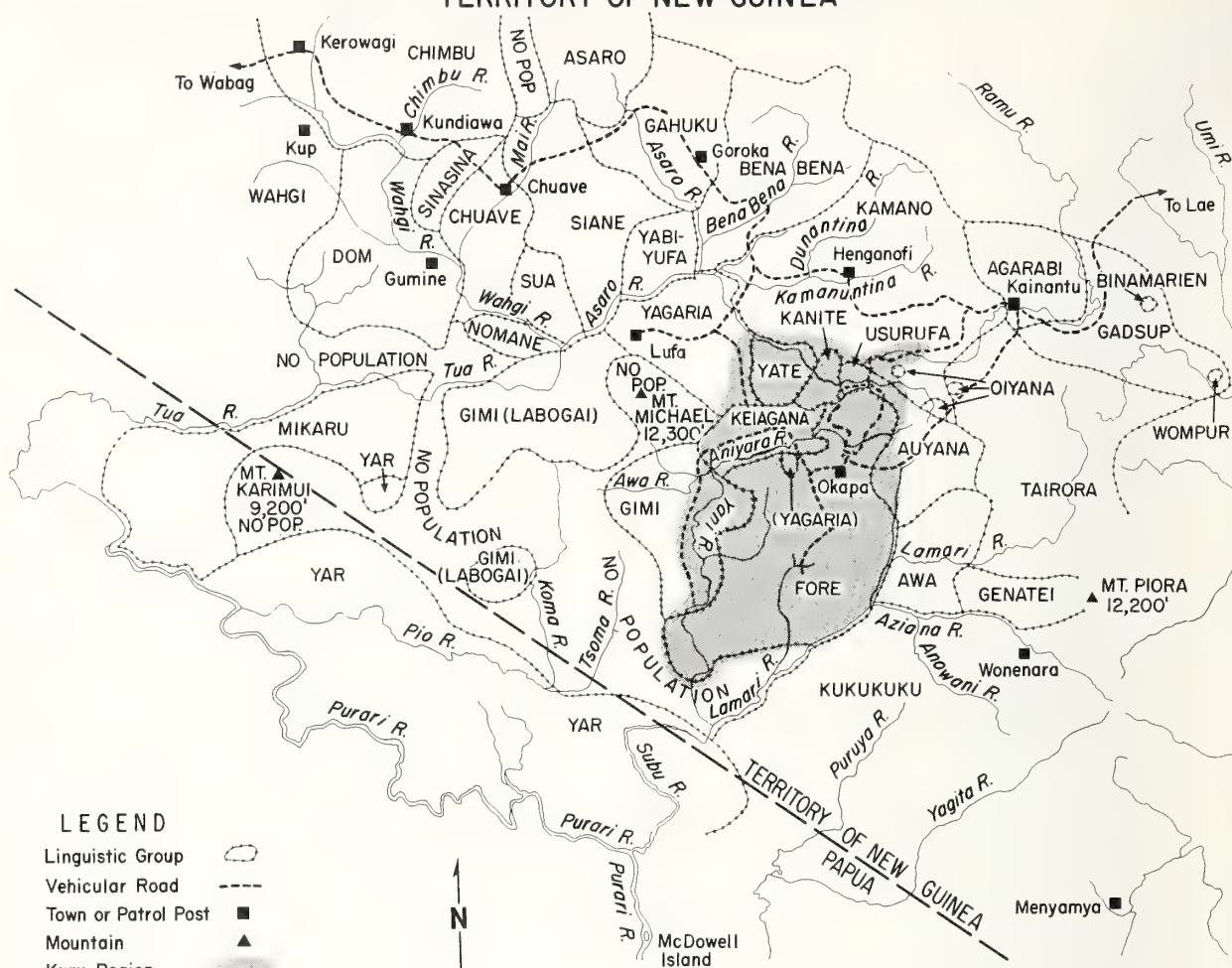


FIGURE 2.—(After Curtain, Gajdusek, and Zigas (13).)

Apart from being a neurological disease showing a strikingly high incidence in a limited and remote area, which is a highly unusual but not unique phenomenon, kuru is a unique disease considered purely from the clinical and pathological points of view. These aspects are dealt with by Drs. Gajdusek and Klatzo, Mrs. Beck and Miss Neumann in their papers of this symposium (1-4).

Kuru is said to have begun as a recognizable fatal disease, in the form in which it is found at the present time, about 40 or 50 years ago. It is said to have started in one hamlet and spread fairly slowly but steadily throughout the kuru region. This is the history consistently given by native informants. Robert and Shirley Glasse, anthropologists who worked in the kuru region, have followed up the local accounts of the origin of the disease very carefully and have found a remarkable congruence between the first hand

accounts of many independent informants. Though kuru is one of the most important features of the life of the region, and sets these people apart from their neighbors, they have not developed any traditional means of coping with the disease, or with the infants who are so often rendered motherless by it, beyond including it among the sorceries which are practiced among them (5). It is true that kuru sorcery embodies an elaborate system for its invocation and an equally elaborate counter system for the detection and punishment of the sorcerer and cure of the patient; but sorceries (without there being necessarily a one:one correspondence between forms of sorcery and forms of disease) have been found to increase and decline very rapidly, so that the presence of or degree of complexity in a type of sorcery cannot be regarded as evidence for the time of origin, either ancient or recent, of the underlying disease. The social argument in regard to



FIGURE 3.—Map of the kuru region showing the position of every village with a history of kuru. The linguistic group is indicated after each village name: FS South Fore, FN North Fore, G Gimi, KE Keiagana, KN Kanite, Y Yate, A Auyana, U Usurufa, YA Yagaria, KM Kamano. The names of the “tribal” groups are printed in *italics* (see figs. 6 to 9 for the most precise boundaries of these groups). (After Gajdusek, Zigas, and Baker (11).)

AGE DISTRIBUTION OF KURU DEATHS

1957-1959 and 1961-1963

by two different age sortings

1957-1959

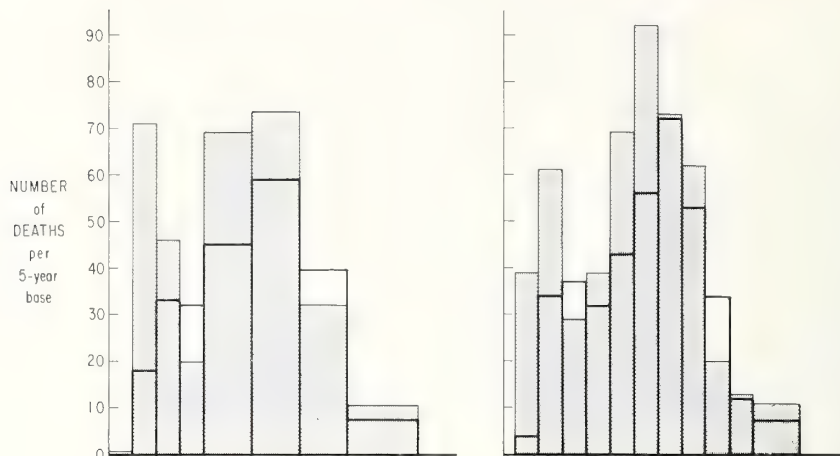
1961-1963

= 1 death

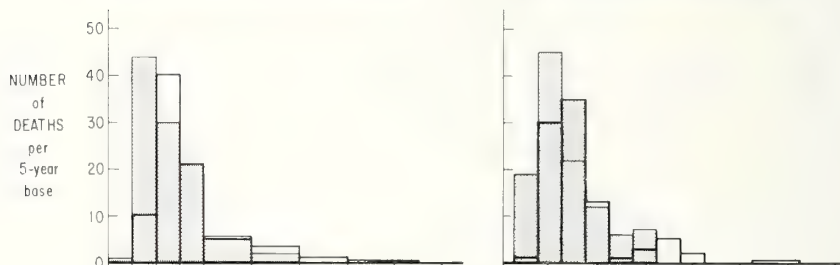
DISTRIBUTION #1

DISTRIBUTION #2

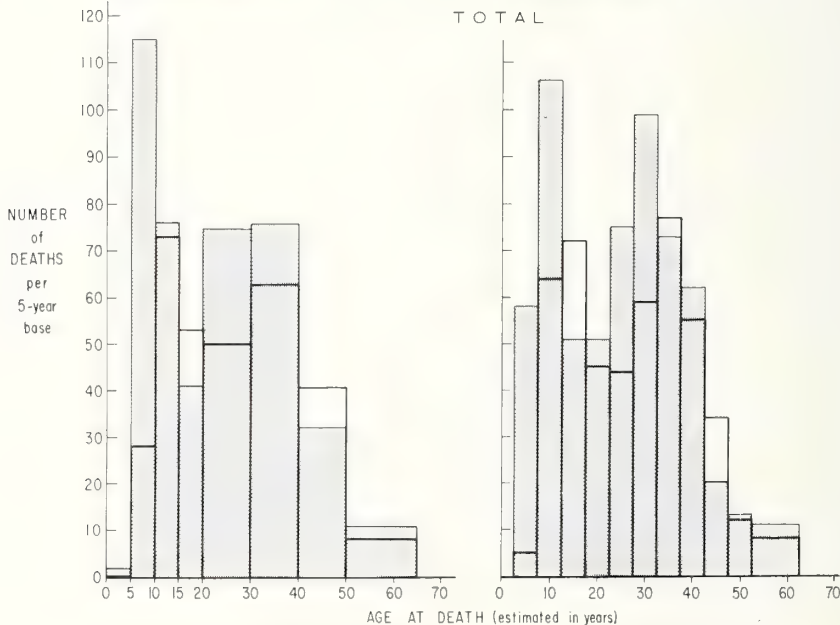
FEMALE



MALE



TOTAL



AGE AT DEATH (estimated in years)

FIGURE 4.—Distributions No. 1 and No. 2 are derived from the two different age sortings that were used. They demonstrate that the age distribution cannot be explained merely as the combined result of bias in the age estimation and the 5-year intervals chosen for the distribution. (After Alpers and Gajdusek (9).)

the infants may have a little more cogency, at least from a European's viewpoint, but can provide only circumstantial evidence for the recent origin of the disease. The case for this must therefore rest with the verbal testimony of local informants. Though the evidence is impressive, the period in question is at about the limit of informants' memories and this, coupled with the fact that these people have no method of reckoning time, casts some doubt on the reliability of these accounts (6). In these circumstances the question as to whether kuru is of relatively recent origin or not becomes impossible to resolve conclusively.

If we must keep an open mind about this matter, we do know that it was not until the early 1950's that the disease came to the notice of European observers (see Berndt (7), Julius (8)), and only in late 1956 did medical examination of kuru patients begin. In early 1957 Dr. Gajdusek joined Dr. Vincent Zigas in the field and in the next 12 months, working day and night, they methodically plotted the boundaries of the kuru region, and with the assistance of the government officer, Mr. Jack Baker, and a system of medical orderlies and native informants, accounted for every case occurring within it. In addition, they set up a hospital and research center in Okapa, made careful clinical studies of many patients from throughout the region, and obtained postmortem tissue, blood, urine, and cerebrospinal fluid for pathological, biochemical, serological, and trace metal studies. From the end of 1957 a research team from the University of Adelaide had joined them in the field and worked on amassing genealogical data, from which they were able to propose a genetic model for the disease, as well as contributing to the clinical and pathological studies. Since that time, fairly continuous investigation into the disease has been going on, with periodic visits of medical investigators, as well as regular demographic patrols.

We have recently examined the records collected over this period, amounting to more than 1,450 cases, firstly to recheck them all for errors and inconsistencies, and then to compare the epidemiological pattern of kuru as it was when the disease was first discovered with that found in more recent years (9). A 7-year period, 1957 through 1963, in which complete mortality figures had been collected, was at that time available for study and the first 3 years of it, 1957 through 1959, were compared to the last 3, 1961 through 1963. In addition, the figures were analyzed by year to see whether the changes found fitted into a continuous trend. The analysis was made in terms of mortality and mortality rate for the whole kuru region and then

separately by linguistic group, "tribal" group¹ and, finally, by village.

RESULTS

The sex and age distributions of all deaths from kuru in the two 3-year periods are compared in figure 4. The comparison has been made using two distinct age sortings (based on 5-year intervals beginning at 0 and 2½ years respectively), in order to allow for the possibility of spurious differences arising from biases in the estimation of age.² The distributions from the two sortings are found to be entirely consistent and show that a striking reduction in childhood deaths from kuru took place between the two periods. This was even more marked in younger children than in older, as the second distribution shows, and occurred in both males and females. In 1957 to 1959 there were 117 cases under 10 years of age, while in 1961 to 1963 only 28. Using the second distribution there were 58 cases under 7½ (or 8) in the early period, only 5 in the later; 106 cases between 7½ and 12½ in the early, 64 in the later. There was, over all, relatively less reduction in mortality in the adult age groups, and among adolescents there was an apparent slight rise. When all cases are considered, and related to the population in 1958 and 1962 respectively, the mortality rate in deaths per thousand population per annum fell from 7.6 to 5.6. Total population of the kuru region, which is now of the order of 40,000, showed over the period under study a steady increase of just over 2 percent per annum; even in the South Fore, where the incidence of kuru is at its highest, there was a natural increase in the population over this period. Unfortunately, it is not possible to construct reliable age specific mortality tables with the population figures available.

Tabulation of the total mortality from kuru by separate year shows that the number of deaths fell fairly steadily with time; and both the number for the year excluded by the two 3-year periods, 1960, and that for the following year, 1964, fell into the same general pattern of decline (table I). The relatively greater reduction in child mortality is further emphasized when child and adult deaths are plotted

¹ "Tribal" group is the term used by Gajdusek (10) for the natural subdivisions of each linguistic group and is equivalent to the term "native area" used in an earlier paper (11). There is no true tribal organization among the people of this region, but their effective units of social structure, roughly equivalent to villages, do combine to form larger groups which have been designated, for want of a better term, "tribal". These groups still have meaning in the native context, but are not used for administrative purposes.

² See footnote 2 on following page.

TABLE I.—Total Kuru Mortality by Year, 1957 to 1964

Year:	Total deaths from kuru
1957 -----	203
1958 -----	209
1959 -----	218
1960 -----	186
1961 -----	169
1962 -----	151
1963 -----	163
1964 -----	117
-----	-----
Total -----	1416

It is not possible to calculate accurate mortality rates for these figures because of the lack of population data for the whole population at risk in each year, but it is known that in all areas of this population there has been a natural increase throughout the period under study.

separately for each year from 1957 to 1964 (fig. 5). The child:adult ratio of deaths declined from 0.46 in 1957 (with a peak of 0.52 in 1959) to an estimated 0.13 (final figure 0.15) in 1964. Male and female deaths are also plotted: male deaths showed little over-all change, whereas female deaths declined; however, the male: female ratio of deaths, though it fluctuated, did not significantly alter.

It is important next to see how these changes varied, if at all, throughout the different groups and areas which comprise the kuru region. Mortality rates for

kuru, population changes by language and "tribal" group, and the change in the male:female ratio of the "tribal" populations of the region are shown in figures 6 to 9. In figure 10 the region has been divided, not according to cultural and linguistic divisions, but according to village kuru mortality, and the areas of high mortality are compared for the two 3-year periods. In general, it would appear that the area of high kuru mortality has contracted. In two places, however, there occurred a slight expansion of the high mortality area, as defined in figure 10, namely, in the Kamikina group of the South Fore and the Hepavina group of the Gimi. Also, when all "tribal" groups were studied for changes in their total mortality rate between the two periods, these two groups were the only ones to show a significant increase. In the light of this, the high mortality area was looked at more closely to see if the area of increased mortality could be mapped out more closely. A marked discontinuity was found in the high mortality area for 1961-63: in the central part of this area (No. 2 in figure 10) the mortality rate fell markedly (from 25 to 16 per thousand per annum between the two 3-year periods), whereas, in the outside areas No. 1 and No. 3 the mortality rate increased, and this increase, though small, was in fact evident in nearly every village of these parts. The finding of this clear-cut discon-

² To explain this more fully, as an example consider distribution No. 2, which actually divides as in the first line of figures. The remaining figures are purely hypothetical to illustrate how the use of two distributions might disclose a hidden bias.

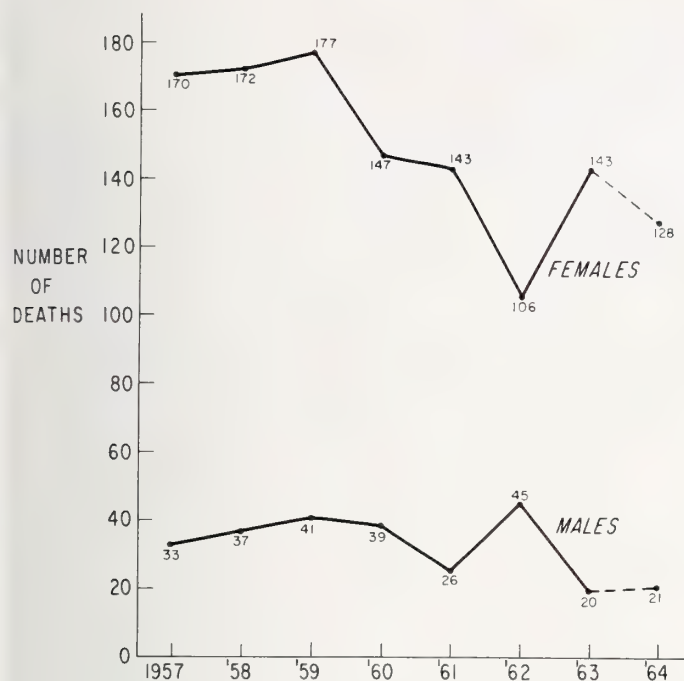
Age	{ 2½	5	7½	10	12½	15	17½	20	22½	25	27½	30	32½
					xxxx	yyyy							
5-year deaths (distribution No. 2)		39		61		29		39		69		92	
Comprised of (hypothetically)		19 20		22 39		6 23		27 12		40 29		46 46	

Distribution No. 2 shows a bimodal distribution. However, if age group xxxx had been overestimated from bias at the expense of yyyy (as in the example given), another distribution of the same figures would show:

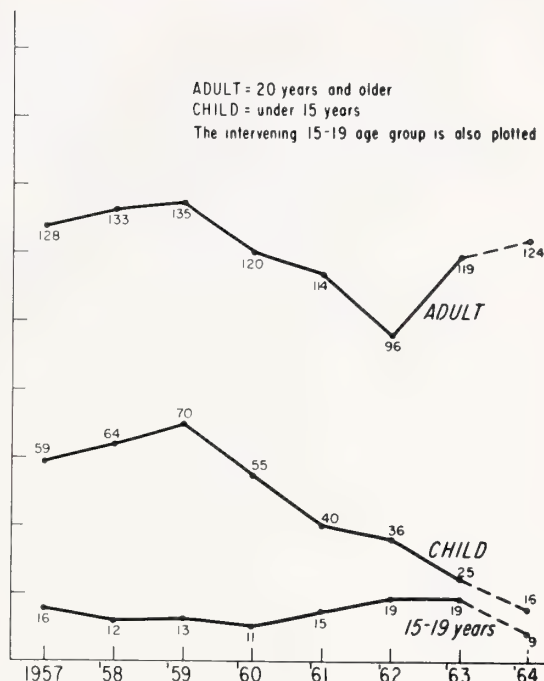
Redistribution would give	19	20 22	39 6	23 27	12 40	29 46	46
5-year deaths	19	42	45	50	52	75	

This distribution clearly has only a single peak and the bimodality has been lost or obscured. It was the product of bias in age estimation and the distribution used. Thus, another distribution, using a different age sorting, has converted a marked bimodal distribution, with two distinct peaks and an antimode, into one with only one peak. No such result occurred with our figures, both distributions being essentially the same.

TOTAL MALE and FEMALE MORTALITY
FROM KURU by YEAR from 1957 to 1963
with the estimated number of deaths for 1964



TOTAL ADULT and CHILD MORTALITY
FROM KURU by YEAR from 1957 to 1963
with the estimated number of deaths for 1964



CHILD : ADULT and MALE : FEMALE RATIOS OF ALL DEATHS
FROM KURU by YEAR from 1957 to 1963
with an estimate of the ratios for 1964

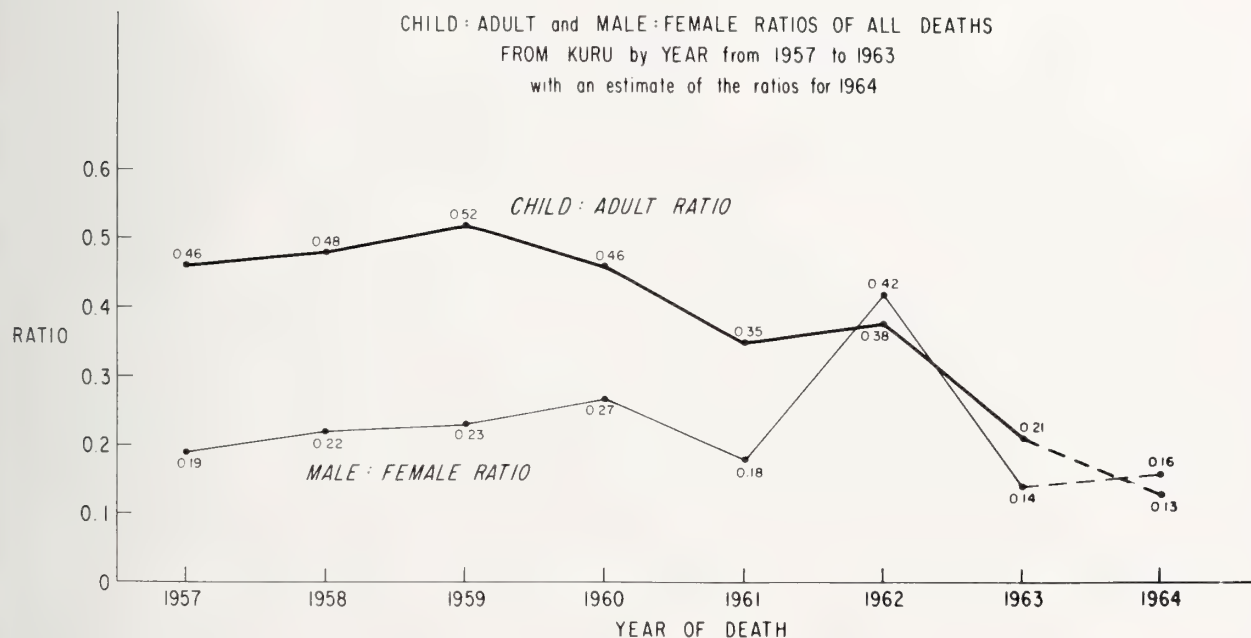


FIGURE 5.—Kuru mortality and mortality ratios by year from 1957 to 1963, with an estimate for 1964 based on information available in December 1964. (After Alpers and Gajdusek (9).) The estimate proved to be too high, final figures for 1964 being female deaths 97, males 20; adult deaths 93, children 14, adolescents 10; child : adult ratio 0.15 and male : female ratio 0.21.

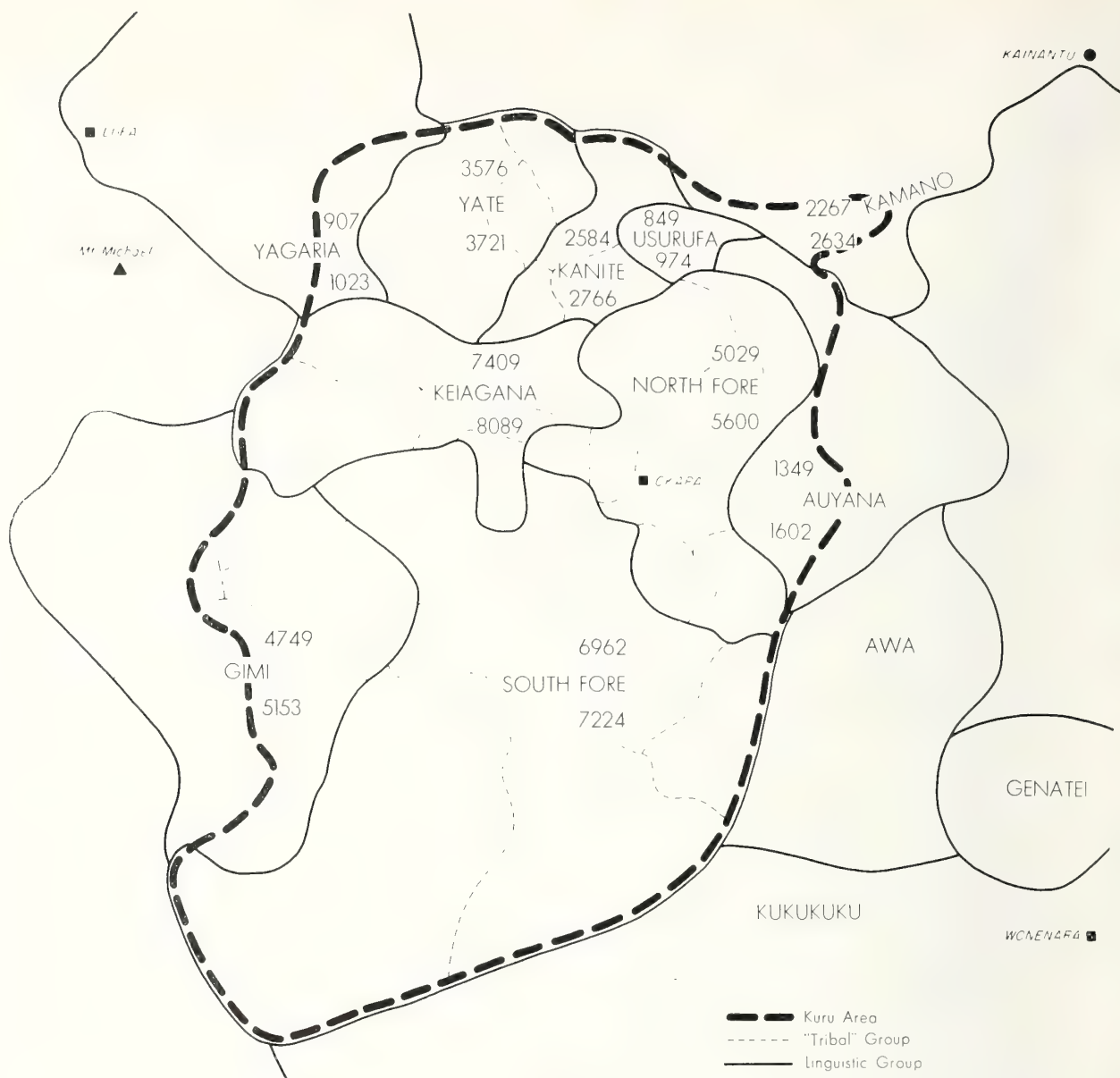


FIGURE 6.—Population of linguistic groups of the Kuru region, 1958 and 1962. The figures for peripheral groups include the populations of only those villages with a history of Kuru (see fig. 3) and do not represent the total populations of these groups. The numbers above each name refer to 1958, those below to 1962.

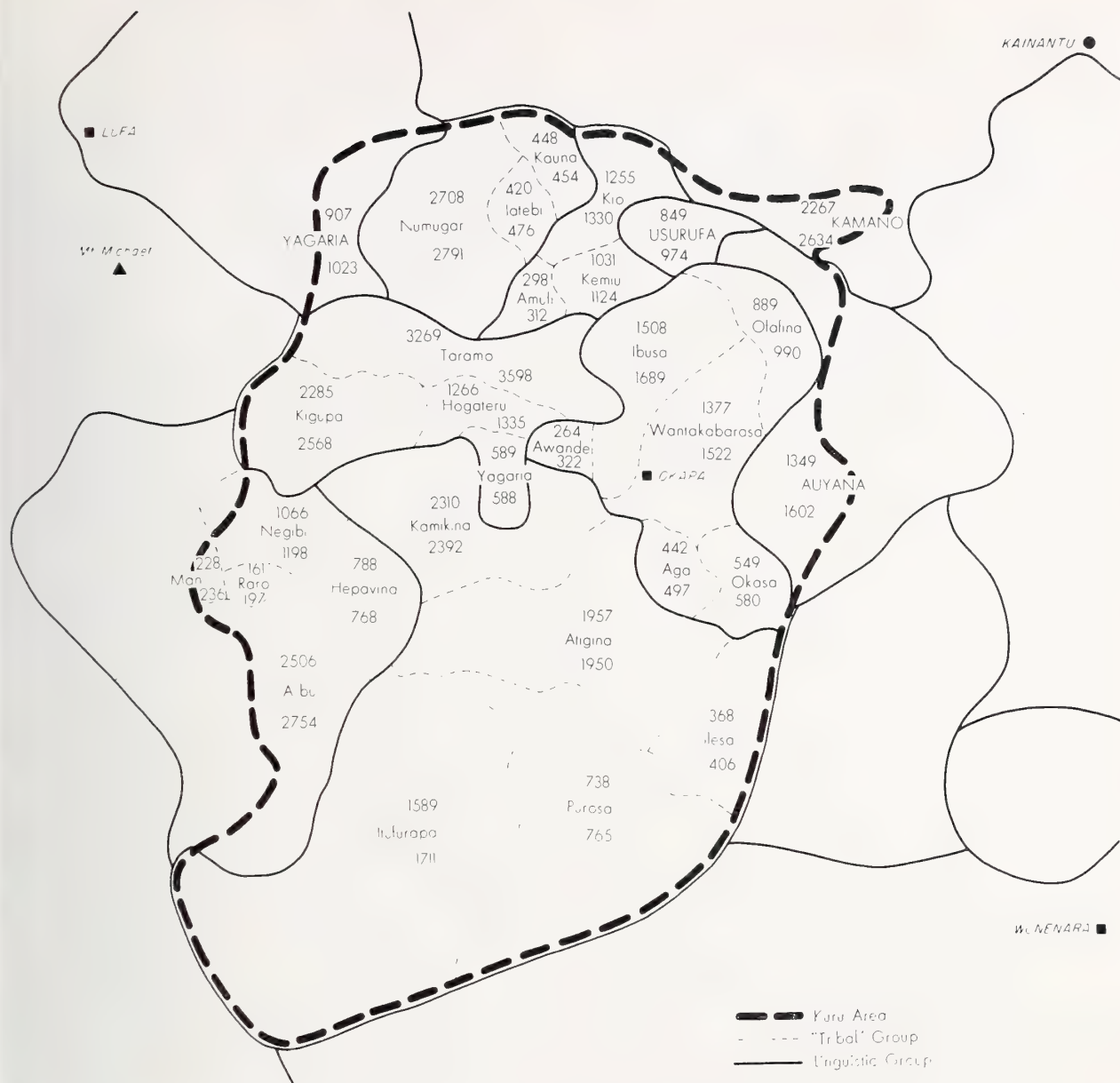


FIGURE 7.—Population of "tribal" groups of the kuru region 1958 and 1962. The figures for peripheral groups, most of which are in fact linguistic groups (names printed in capitals), include the populations of only those villages with a history of kuru (see fig. 3) and do not represent the total populations of these groups. The numbers above each name refer to 1958, those below to 1962.

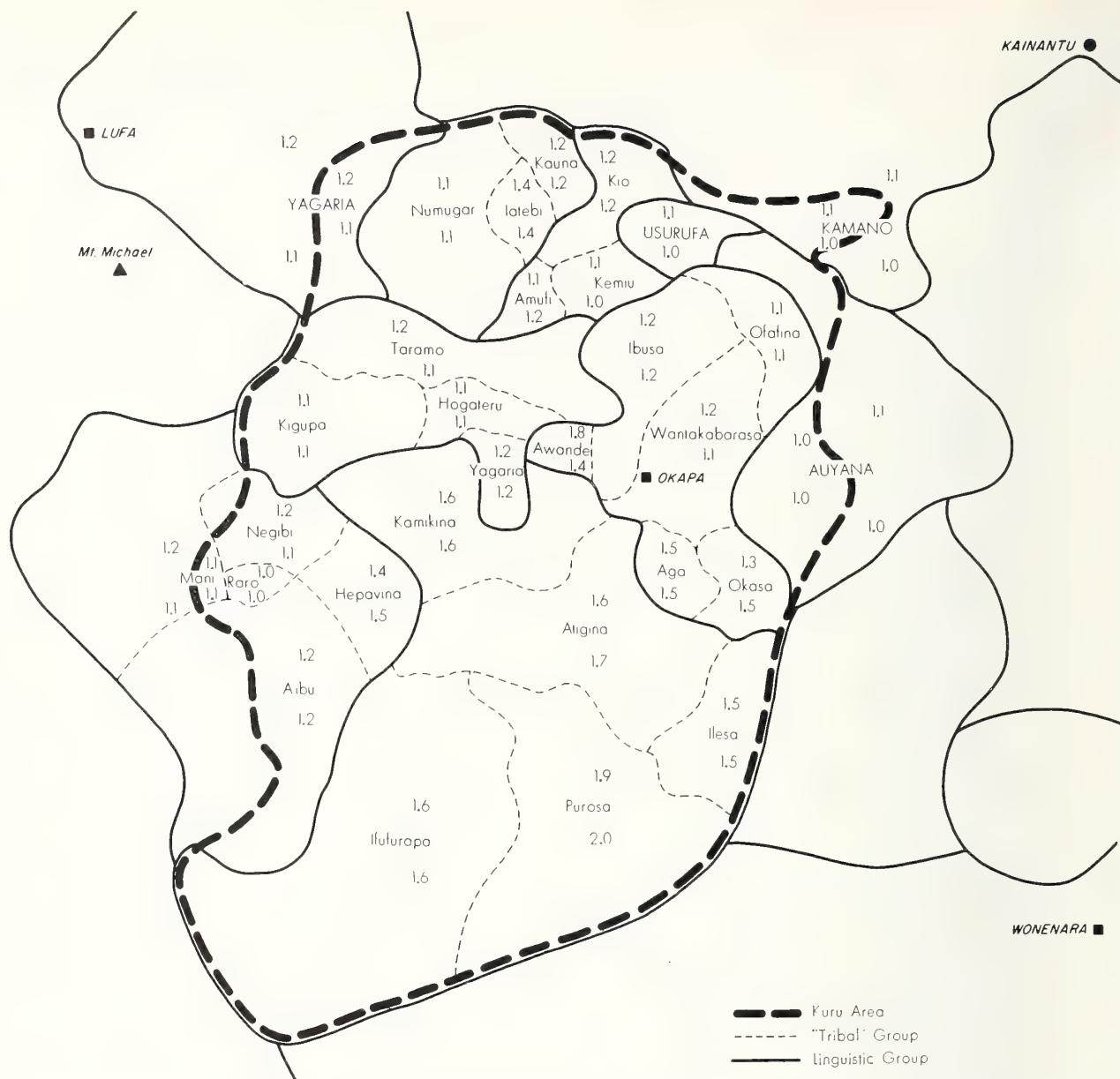


FIGURE 8.—Male: female population ratio in each “tribal” group of the kuru region, 1958 and 1962. The two sets of figures for peripheral groups refer to their portions within and without the kuru region. The ratios above each name refer to 1958, these below to 1962.



FIGURE 9.—Kuru mortality rate in deaths per thousand population per annum in each "tribal" group of the kuru region, 1957-59 and 1961-1963. The numerators of the rates are obtained from the deaths which occurred in the two 3-year periods, the denominators are the populations of figure 7. The rates above each name refer to 1957-59, those below to 1961-63.



FIGURE 10.—Map showing the “high mortality” areas for kuru, 1957–59 and 1961–63; they are defined as the minimum areas beyond which the kuru mortality rate by village was less than 10 per thousand per annum. The area for 1961–63 has been further divided into three: areas No. 1 and No. 3 where kuru mortality uniformly increased from 1957–59 to 1961–63, and area No. 2 where it markedly decreased. For the names of the “tribal” and linguistic groups involved see figures 6 and 7. (After Alpers and Gajdusek (9).)

CALENDAR MONTH OF ONSET OF 376 CASES OF KURU
JUNE 1956 - MAY 1963

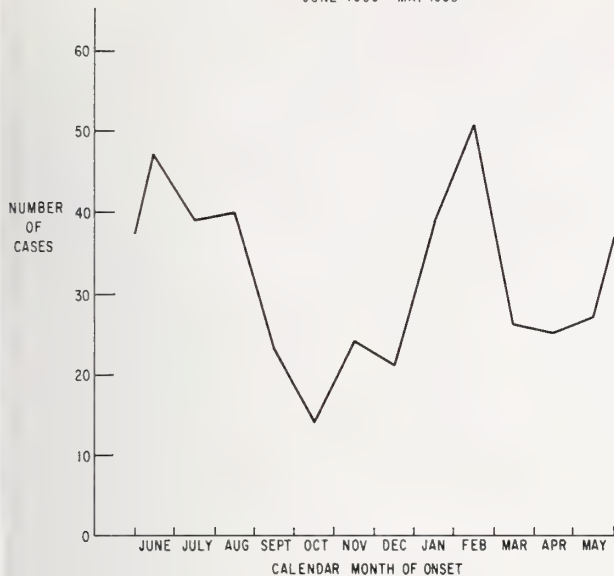


FIGURE 11.—The total number of cases with onset in each calendar month is obtained by summation over the 7-year period and plotted at the midpoint of the month. (After Alpers and Gajdusek (9).)

tinuity came as a surprise and it continues to remain unexplained.

A study of the times of onset and duration of the disease was made in all cases in which this had been documented. There was a suggestion that the duration of the disease had increased in adults in the later period (9). The seasonal onset is shown in figure 11 and for comparison the rainfall figures at Okapa over the same period are plotted (fig. 12). The fluctuation in the second of these figures came as more of a surprise than the fluctuation in the first, but I do not propose to relate them nor to draw any conclusions from these figures beyond the simple fact that in the sample of cases where this information was available (376 out of 1,300 between 1956 and 1963, and in this sample there is no obvious bias), there was considerable seasonal fluctuation in the documented time of onset of kuru.

DISCUSSION

I do not propose to discuss these results in detail at this point. The general conclusion of the decline in kuru mortality, especially among children and most marked among the younger children, is inescapable (and I hasten to add that the case fatality rate has not dropped at all, but remains at 100 percent). The present decline is the second most important fact in the epidemiology of kuru. The evidence presented, in

MONTHLY RAINFALL AT OKAPA
JUNE 1956 - MAY 1963

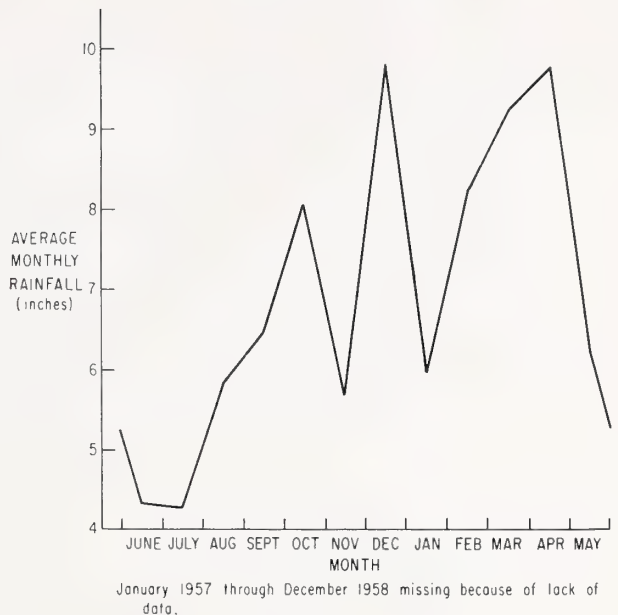


FIGURE 12.—The recorded rainfall at Okapa for each calendar month is summed over the 7-year period (though 2 whole years, 1957 and 1958, are missing) and the average plotted at the midpoint of the month. (This data made available by courtesy of the administrative officer at Okapa.)

general, also strengthens the conclusion that an environmental factor is involved in the etiology of kuru. However, the most important epidemiological fact is still the absolute ethnic limitation of the disease to the Fore and the people with whom they have intermarried. The working hypothesis that I and others before me have considered most likely is that an environmental factor for kuru is superimposed on a specific genetic background, long established as a polymorphism among the Fore people. The details of this genetic mechanism remain in doubt, though one model has been proposed (12); and the reason why a mutation, arising at some time during the period of isolation necessary for the Fore language to evolve, became established as a polymorphism in this population is unknown. So, too, is the specific environmental factor which has triggered off the disease, but clearly the present decline in the disease could be the result of a decline in this same factor from the environment. It could also in part result from the removal by death of genetically susceptible individuals from the population and the inevitable decrease in the number of such individuals in the next generation. In an attempt to

clarify our own thinking, and as a possible basis for speculation for others, Dr. Gajdusek and I have prepared a table of known environmental changes that have occurred in the kuru region (table II). This aims at being complete as regards things known, and

so we will probably always have to keep adding to it or have others add to it; but, in any case, if none of these known environmental possibilities are sufficient, you can readily turn in your thinking to things unknown—even, for example, to a slow virus.

TABLE II.—Known Environmental and Cultural Changes in the Kuru Region Tabulated According to an Approximate Chronology

[D. C. Gajdusek and M. P. Alpers]

Years	Events	Introduction Into Environment of	Disappearance or Decline of
1900–30	European influence on the coast, with no knowledge of the existence of Highland populations.	The sweet potato may have been first introduced in this period. ¹	
1917–18	Detzner walking among the Kuku-kuku.	Cannibalism: believed to have appeared in the kuru region around 1920.	
1929	Rowlands enters Kainantu area.	Some trade items and possibly maize may have come in through the traditional trade routes.	
1930	Leahy and Dwyer enter the Highlands, pass by Mt. Michael and down the Purari River.		
	Taylor and Leahy patrol into the Highlands.		
1930's	Early miners and prospectors Planes flying over the Highlands. First patrol posts in the Highlands.	Maize and the casuarina (<i>ya</i>) tree: present in the late 1930's.	
1936	First European into the kuru region proper: Ted Ubank.		
Late 1930's	Father Tufanel from Gailala into the Highlands.		
Early 1940's	Japanese war: Australian observation posts in the Highlands; Japanese infiltration from the Markham Valley. Many planes flying over the Highlands and some of them crash, with survivors in some cases making their way through to the coast.		
1943	Dysentery epidemic throughout the Highlands.		The introduction of sweet potato is associated with decreased use of taro and banana in the staple diet.
Late 1940's	First postwar patrols. First patrols into the kuru region.	Newer varieties of sweet potato ¹ —late 1940's and continuing through the 1950's.	
1945–50	Certain southern South Fore communities move south into uninhabited malarial forest.	Trade salt (sodium chloride) introduced: late 1940's and early 1950's. Steel axes: late 1940's and early 1950's.	Stone axes: late 1940's and early 1950's.
1949	Tarabo Mission established (native evangelists). Okasa pine forest surveyed. Usurufa and parts of North Fore declared derestricted (controlled).		
1950	First residing European missionary at Tarabo.		
1950–55	Return and death of southern tip South Fore communities.		

See footnotes at end of table.

TABLE II.—Known Environmental and Cultural Changes in the Kuru Region Tabulated According to an Approximate Chronology—Continued

Years	Events	Introduction Into Environment of	Disappearance or Decline of
1951	Police patrol post at Okapa (Moke village).	Domestic fowl: late 1940's and early 1950's, not reaching outlying parts until 1957.	Cannibalism: 1950-57.
	Government patrol post at Tarabo.	New vegetables (potatoes, tomatoes, cucumbers, cabbage, lettuce, pumpkin, peas, haricot beans, etc.): early 1950's.	Suckling of piglets: 1951-56.
	North Fore declared derestricted (controlled).	New varieties of maize: early 1950's Sweet bananas: early 1950's. Lemons: early 1950's.	
1952	R. and Catherine Berndt working among Yate, Usurufa and North Fore.	Twist tobacco: early 1950's.	
1953	New Tribes missionary at Yagana in the Keiagana.		
1954	European patrol officer at Okapa.	European drugs, in particular penicillin: 1954. Horses at Tarabo: 1954.	
1955	North Fore laborers in Kainantu, Goroka and Bena Bena.	Coffee: 1955. Passion fruit, pineapple and paw-paw: 1955-56.	Child marriage: discouraged since 1955.
1955	Mage (North Fore) village school established.	Trade salt (sodium chloride) becomes an important part of the diet: 1956.	Native salt (potassium and calcium salts) declines in use and disappears by 1962.
1956	S.D.A. Mission station established at Okasa.	Ducks: 1956. Goats: 1956. Cats: 1956. Cows at Tarabo: 1956; not at Okapa until 1963.	
1956	First North Fore native to see the coast, taken by the Administration.	Kerosene: 1956. Matches: 1956.	
1956	Gonorrhea epidemic, North Fore.	Shovels: 1956.	Digging sticks: not eliminated but use declining since 1956-57.
1956	First plane lands at Tarabo airstrip.	Yellow and red body paint and beads as trade goods: 1956.	Intervillage warfare: 1956-57.
1957	Kuru Research Center set up in Okapa and medical study of kuru begins.	Hospital medical care: 1957. Western clothing and blankets: 1957.	Fighting stockades gone by 1958. Change to family households from the village men's house and change to square houses from the traditional round form: 1957. Yaws eradicated with penicillin: 1957.
1957	Whooping cough epidemic.	Gasoline: 1957. Insecticides, especially D.D.T.: 1957.	Close contact with pigs in the houses: declining since 1957.
1957	First South Fore native to see the coast. Two kuru patients taken to Port Moresby.	Soap: 1957.	Dietary taboos: declining since 1957.
	First Fore to leave the main island, taken to Rabaul.	Rice, tinned meat and other trade foods: 1957. European types of pig and domestic fowls: 1957.	Eating of rodents: declining since 1957.
1957-60	Establishment of medical aid-posts throughout the kuru region.	Peanuts: 1957.	Nasal cane passing: 1957-58.
1958	World Mission station established at Purosa.	Boiling of food associated with increasing use of utensils for cooking: 1958.	Ritual killing (<i>tokabu</i>): 1958.
	South Fore, southern Keiagana and Gimi declared derestricted (controlled).		Use of nettle as a counterirritant: 1958. Smoke cure ceremonies: declining since 1958.

See footnotes at end of table.

TABLE II.—Known Environmental and Cultural Changes in the Kuru Region Tabulated According to an Approximate Chronology—Continued

Years	Events	Introduction Into Environment of	Disappearance or Decline of
1958-59	Government school established at Okapa.		Yellow and red body paint and beads as trade goods: 1958.
	Lutheran evangelists move into South Fore.		Leprosy: treatment begun in 1958, virtually under control by 1964.
1959	Negibi (Gimi) Mission established.		Use of twist tobacco as a trade item: declining since 1959.
1959	First Fore laborers sent throughout New Guinea.		Pig grease covering: 1959.
1959	Money economy begins in the kuru region.		Nose piercing: 1959.
1959	Trade stores open in Okapa. ²		Taro and edible pandanus in the diet: declining since 1960.
1959	Asian influenza epidemic.		Chewing of bark: 1960.
1960	S.D.A. Mission school established at Keiakasa (South Fore).		
1961	First Fore laborers sent to outer islands of New Guinea.		
1962	Coffee Training School at Okapa.	Tea drinking becoming common in the villages: 1962-63.	
1962	New Government hospital completed at Okapa.	Alcoholic drinks freely available, though their use did not extend to the villages: 1963.	
	Lutheran Mission hospital for kuru patients established at Awande.		
1964	First elections for Legislative Council.	Triple antigen immunization of children: 1963.	
1965	Local Council being established.	Sheep tentatively introduced: 1965.	
		Animals and foods still in use and believed to be of long-standing origin in the region: pig (native variety), dog, wild game; taro, yam, manioc, banana (cooking variety), sugarcane, native greens, edible pandanus, edible pitpit.	

¹ From the evidence of Watson among the Tairora and Gajdusek among the Kukukuku that the first introduction of the sweet potato into these groups occurred in recent years, its first introduction into the adjacent kuru region may also be more recent than commonly believed (see 14).

² Though trade store items were available at Tarabo before this date and many trade items entered the region with patrols they did not become generally available until the trade stores opened in Okapa.

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DISCUSSION

BURGER: If you were to plot high and low incidence regions separately, would you get similar pictures in the sex and age distributions?

ALPERS: The distributions do vary. The complete distribution breakdown is unrewarding where there are small numbers, but by using the child: adult and male: female ratios it is possible to see how the general distributions change with mortality rate (fig. 13). As one gets into areas of low incidence, the child:adult ratio of cases drops markedly, and there is a close correlation between the child:adult ratio and the mortality rate, which is interesting in view of the fact that the child cases were considered to be the homozygotes for the kuru gene. The male: female ratio, on the other hand, does not change with mortality rate.

POSKANZER: This age and sex distribution you have described is almost exactly that of pellagra as described by Goldberger in the American South: virtual absence of cases in adult males, the bimodality among females and the large number of cases in children. The breadwinner in the family always was fed better. I think that it is an interesting analogy because this is a very unusual pattern for an age and sex distribution for any disease. It reminded me immediately of pellagra.

HAIG: This decrease in the onset among children, is this correlated in any way with the time when they stopped eating flesh?

ALPERS: It is believed that the Fore started to cannibalize their dead only about 40 or 50 years ago, which is also about the time they maintain kuru started, though they themselves do not relate the two. They apparently heard that human flesh was good to eat, they tried it and they liked it immensely and continued to eat it until the administration came

and discouraged the practice. Among the Gimi even now there may be a little bit of cannibalism going on, for they have been the slowest to change, but among most of the people of the kuru region there has been no cannibalism since 1957. The children are certainly not eating human flesh; the adults may well have eaten it as children and that may have been sufficient to cause them still to get the disease. I do not wish to speculate in detail at this point, but it is clear that of the many environmental changes in the kuru region cannibalism is one which very neatly explains a lot of the phenomena that we have been observing; however, despite the fact that it was, I think, Dr. Gajdusek's first hypothesis, I do not think we should give it any other priority among the hypotheses for the etiology of kuru.

CASALS: Are these the only people who practiced cannibalism in New Guinea?

ALPERS: Definitely not. Nor are they the only people who practiced cannibalism in this area of New Guinea. The Fore are said to have learned the practice from the Kamano.

HOTCHIN: Do other neighboring groups eat the Fore, including healthy Fore? It is possible that the Fore carry an agent even though it has not yet produced the disease.

GAJDUSEK: Before 1957 natives living outside of it had never seen a person from the kuru region—except from the immediate periphery, but in most of the peripheral villages the incidence of kuru drops very markedly.

PATERSON: What is the temperature at which the human flesh was prepared?

GAJDUSEK: Usually inadequately cooked. The brain was also always consumed and not only is there the intriguing immunological possibility of hypersensitization to brain antigens arising from this but there is the further immunologic point of "adjuvant" added, namely, they often waited until the third or fourth day when putrefaction had occurred before consuming the dead. I do not believe that cannibalism is the answer, but if one wants to consider all aspects of it, the Fore did often eat partially cooked brain which was contaminated with putrefying flesh and viscera. We looked for antibrain antibody early in the disease in sera from kuru victims, by many types of reactions, but found nothing. We began to doubt the importance of cannibalism, too, when we found cases of kuru in children who had been reared on the police station from birth and had never been near a cannibalized dead body.

It is possible that they went off surreptitiously of an evening and consumed some human flesh, but we doubted this very much, and thus we were forced to say that if cannibalism had anything to do with the disease it could only have introduced hypersensitivity in some individuals one generation back which was then transmitted vertically to offspring from parents. There is another possibility apart from hypersensitivity: that there was involved a transmissible scrapie-like replicating agent which was transmitted by the oral route from meat, cooked

or uncooked. This could easily be defended, especially if one allowed for transplacental transmission or neonatal infection from the mother so that, even with one generation without cannibalism, the disease might still occur. Certainly, cannibalism has never been omitted from consideration and has been thoroughly studied by the two anthropologists Robert and Shirley Glasse, but we do not believe it is the answer to kuru; the suggestion is more in the nature of an embellishment to other etiological hypotheses.

CHILD:ADULT RATIO OF KURU DEATHS vs. MORTALITY RATE IN 20 'TRIBAL' GROUPS

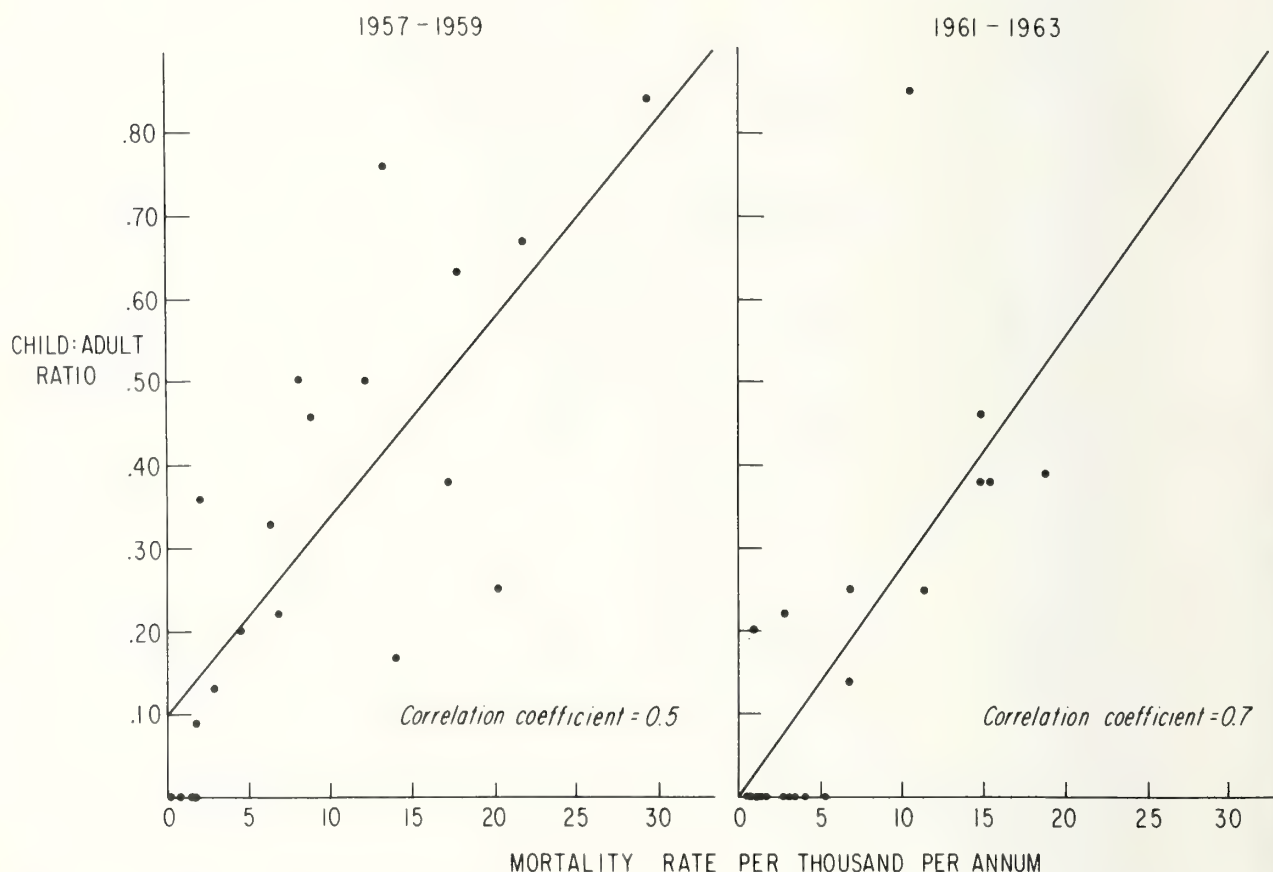


FIGURE 13. Changes in the "age distribution" of kuru deaths with "tribal" groups: correlation between the child: adult mortality ratio and the mortality rate among "tribal" groups. In the later period there is a fall in both these indices, but an increase in the correlation between them. The male:female mortality ratio, which is not shown, is found to show no correlation with mortality rate in either period. (After Alpers and Gajdusek (9).)

Neuropathological Findings in Kuru

IGOR KLATZO

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A new dramatic neurological disorder occurring with high incidence in natives in the restricted area of the Eastern Highlands of the Australian Trust Territory of New Guinea was first reported by Gajdusek and Zigas (1). The disease affects the Fore tribe as well as their tribal neighbors and is called by the natives kuru, which means in the Fore language trembling associated with cold or fear. In adults kuru affects predominantly the women, but in children the distribution between sexes is approximately even. The indications for a high familial incidence are very strong as can be judged from the comparison between immediate families of kuru and those of unaffected natives.

The pathological findings in kuru have been reported first by Klatzo et al. (2) and by Fowler and Robertson (3).

The main pathological alterations on the basis of 12 cases studied in our laboratory were as follows:

The organs other than the central nervous system did not reveal any significant changes. Grossly the brains were unremarkable. Microscopically all cases showed widespread alterations within the central nervous system which consisted in neuronal and myelin degeneration, astroglial and microglial proliferation, and perivascular infiltrations with mononuclear elements. One-half of the cases revealed remarkable plaque-like bodies.

Neuronal changes of nonspecific character were demonstrable from the lowest segments of the spinal cord up to the cerebral cortex. These nonspecific neuronal changes consisted in chromatolysis, pyknosis, hyperchromia or vacuolation. Microglial neuronophagia was quite frequently encountered. Neuronal degeneration varied greatly with regard to intensity and to topographical distribution. Most frequently the severe changes were observed in the pontine nuclei,

basal ganglia and in the cerebellum. In the spinal cord the neuronal changes were generally slight or moderate. In the medulla the inferior olive neurons were frequently affected, whereas the nuclei of XII, dorsal X, and ambiguous were usually resistant. In the pons severe neuronal degeneration was conspicuous in the pontine nuclei, whereas the neurons of the tegmental regions usually revealed only slight changes. In the midbrain the substantia nigra and the red nucleus were occasionally severely involved. The majority of the cases showed intense neuronal degeneration in the basal ganglia and thalamus. In the striatum the large-type neurons showed frequently a moth-eaten appearance. In the thalamus predominantly affected were the anterior and the ventrolateral nuclei. In the hypothalamus the mammillary bodies appeared to be more vulnerable than other hypothalamic nuclei. In the cerebellum the Purkinje cells were usually rather well preserved; on the other hand, there were frequently observed ballooning of the Purkinje cell axons (torpedoes). In some cases there was an obvious reduction in the population of the neuronal elements of the granular layer. Neuronal degeneration was seen in the deep cerebellar nuclei, particularly in the nucleus dentatus. Several cases revealed areas of rather intense neuronal degeneration in the cerebral cortex.

Myelin degeneration was generally not severe and it never amounted to a complete demyelination. Myelin decomposition, which could be best demonstrated by counterstaining with Sudan stains, was almost regularly found in the spinal cord where the corticospinal and spinocerebellar tracts were commonly involved. One-half of the cases revealed a diffuse pallor of the anterior and lateral columns.

Astrocytic proliferation was widespread and intense in the majority of the cases. Although the severity of neuronal degeneration and the intensity of astrocytic

gliosis were usually topographically correlated, some areas showed a conspicuous dissociation in coexistence of these changes. Thus, e.g., the cerebral cortex occasionally revealed an intense astrocytic gliosis without a noticeable counterpart of neuronal degeneration.

Microglial proliferation was extremely widespread in all cases studied. The majority of microgliaocytes showed various forms of reactivity. In the cerebral cortex the rod cells were conspicuous. Generally, the most prevalent form of reactive microglia was of the amoeboid type. Microglial neuronophagy and rosette formations were occasionally encountered.

The blood vessels showed frequent cuffings with hematogenous mononuclear cells and occasional slight thickening of the walls. The cuffings ranged greatly in intensity and they were most frequently observed in the medulla, pons and midbrain. These perivascular infiltrations were mostly of lymphocytic character and they were confined to the Virchow-Robin spaces.

One half of the cases showed the presence of remarkable plaque-like bodies. The histological appearance of these kuru plaques was quite uniform and

consisted of a homogeneous, strongly PAS-positive center surrounded by a delicate ciliated border. The plaques were most frequently found in the cerebellum, although occasionally they could be encountered in the basal ganglia and the cerebral cortex.

The characteristic and basically similar pathological changes observed in our cases allow the recognition of kuru as a new pathological entity. On purely morphological grounds kuru appears to be an acute degenerative process of the central nervous system bearing resemblance to the degeneration of Creutzfeld-Jacob type, especially in the widespread neuronal changes and the lack of any well-defined systemic pattern of the disease.

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Kuru and Scrapie Compared: Are They Examples of System Degeneration?¹

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The first comprehensive description of the pathological changes in the central nervous system of cases suffering from kuru was given as recently as 1959 by Klatzo, Gajdusek, and Zigas (1, 2). In their study of 12 cases the authors were particularly impressed by the diffuse nature of the disease process which affected cerebrum, cerebellum, brainstem and spinal cord, and for which they could find no parallel in human neuropathology. In a further study of three cases Neumann, Gajdusek, and Zigas (3) reached essentially the same conclusions.

Natural scrapie, on the other hand, has been known for a great many years and some of the pathological changes within the nervous system were reported as early as the turn of the century. Over the years various investigators have described widespread and essentially "nonspecific" changes in the central nervous system of sheep affected by scrapie. Recently, however, we found in addition to these a selective involvement of neuronal systems which, we believed, helped to explain some of the clinical features typical of the disease. (For a review of the literature and other details see Beck, Daniel, and Parry (4).)

METHODS

We ourselves have examined 9 cases of kuru, received from Dr. Gajdusek, and 34 cases of natural scrapie. The pathological findings in these two series have been compared with each other and also with those in six cases of carcinomatous cerebellar degeneration.

The material was fixed in 10-percent formol-saline. Representative blocks were taken from the brainstem and cerebellum, the cerebral cortex and spinal cord (where available). The full length of the basal ganglia, hypothalamus, and thalamus was cut into serial sections. A great variety of special staining methods for the selective demonstration of the different parenchymatous elements and their breakdown products was employed.

RESULTS

Kuru²

In all our cases we found widespread "nonspecific" changes in the central nervous system, changes such as various types of neuronal degeneration, astrocytic hypertrophy and status spongiosus. Such changes could be so severe as to obscure the normal architecture of a given region. In addition to the diffuse "nonspecific" changes, however, we were impressed by the selective impact of the disease upon specific neuronal systems, in particular upon the cerebellar system.

The most outstanding feature in the cerebellar cortex was a very severe loss of granule cells and a moderate to severe loss of Purkinje cells (fig. 1A). Of the remaining Purkinje cells many were degenerating as shown by great numbers of torpedo-like swellings on their axons (fig. 2A). Empty baskets were abundant. A dense network of glial fibers had formed throughout all layers of the cerebellar cortex (fig. 3A); in addition the molecular layer contained innumer-

¹ This work was supported in part by a research grant from the Bethlem Royal and the Maudsley Hospitals.

² For the purpose of this paper the nine cases, whose pathology was almost identical, will be described collectively. A more detailed publication is planned in the near future.

able microglial cells in all stages of phagocytic activity. On the whole the degeneration was more marked in the vermis than in the cerebellar hemispheres and within the vermis the nodule was the most severely affected part. Varying numbers of PAS-positive plaques were found within both the molecular and granular layers of six cases (fig. 4A). The cerebellar white matter did not look demyelinated to the naked eye although large amounts of myelin breakdown products were shown up when stains for neutral fat were used (fig. 5A). In addition a dense fibrous gliosis was present throughout. The dentate nucleus showed varying grades of degeneration, its ventral part being usually more severely affected than its dorsal part. In the brainstem many of the nuclei with cerebellar connexions, i.e., the pontine nuclei, inferior olives, medial vestibular nuclei, external arcuate and

external cuneate nuclei, were degenerated, their territory showing a selective dense fibrous gliosis (figs. 6A, 7A). Vacuolated neurones in these nuclei were rare. The middle and inferior cerebellar peduncles, though not visibly demyelinated, showed large deposits of neutral fat. In four of the nine cases the pyramidal tracts appeared degenerated. In the striatum many of the large neurones showed intracellular vacuolation while the small ones always appeared normal. There were also areas of status spongiosus and in these regions the astrocytes were hypertrophic.

The median eminence and pituitary stalk contained large masses of amorphous neurosecretory substance, Herring corpuscles of all sizes and beaded Gomori-positive fibers. There were signs of nerve fiber degeneration in the hypothalamo-neurohypophysial tract although there was no unequivocal evidence of neuronal

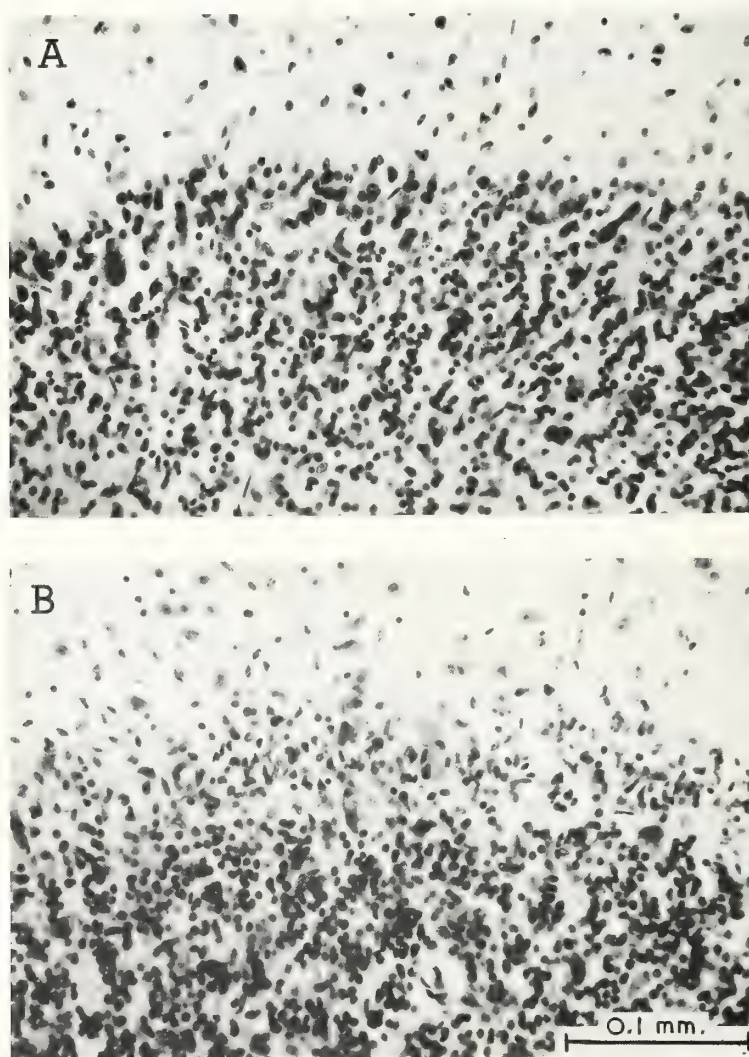


FIGURE 1.—Severe loss of Purkinje and granule cells in the cerebellar cortex. A, Kuru; B, Scrapie. (Nissl stain.)

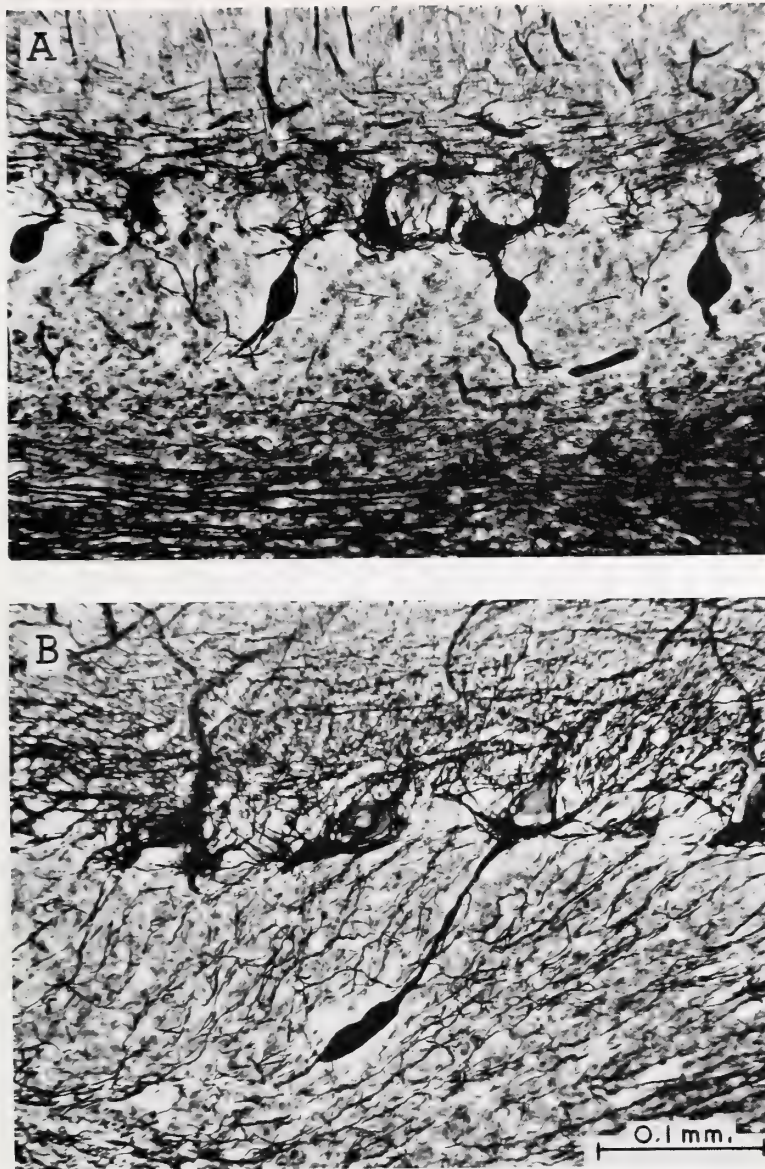


FIGURE 2.—Torpedo-like swellings on axons of degenerating Purkinje cells.
A, Kuru; B, Scrapie. (Gros-Bielschowsky silver impregnation.)

degeneration or glial reaction in the supraoptic and paraventricular nuclei.

Scrapie

As in kuru, widespread “nonspecific” changes were present in all our 34 cases of scrapie, but again as in kuru a degeneration of certain specific neuronal systems was the more striking feature. The systems mainly affected were the cerebellar system and the hypothalamo-neurohypophysial system.

In the cerebellar cortex there was severe loss of Purkinje and granule cells (fig. 1B) with many empty

baskets. There was much nerve fiber degeneration including torpedoes (fig. 2B) and degenerating mossy terminals. Fibrous gliosis was intense in all three layers (fig. 3B). As in kuru, these changes were more marked in the vermis than they were in the cerebellar hemispheres and, as in kuru, the nodule bore the brunt of the process. PAS-positive plaques were found within molecular and granular layers in a number of cases (fig. 4B). The cerebellar white matter showed no obvious demyelination either naked eye or in myelin stained sections. Under high magnification, however, great amounts of degenerating myelin were seen which

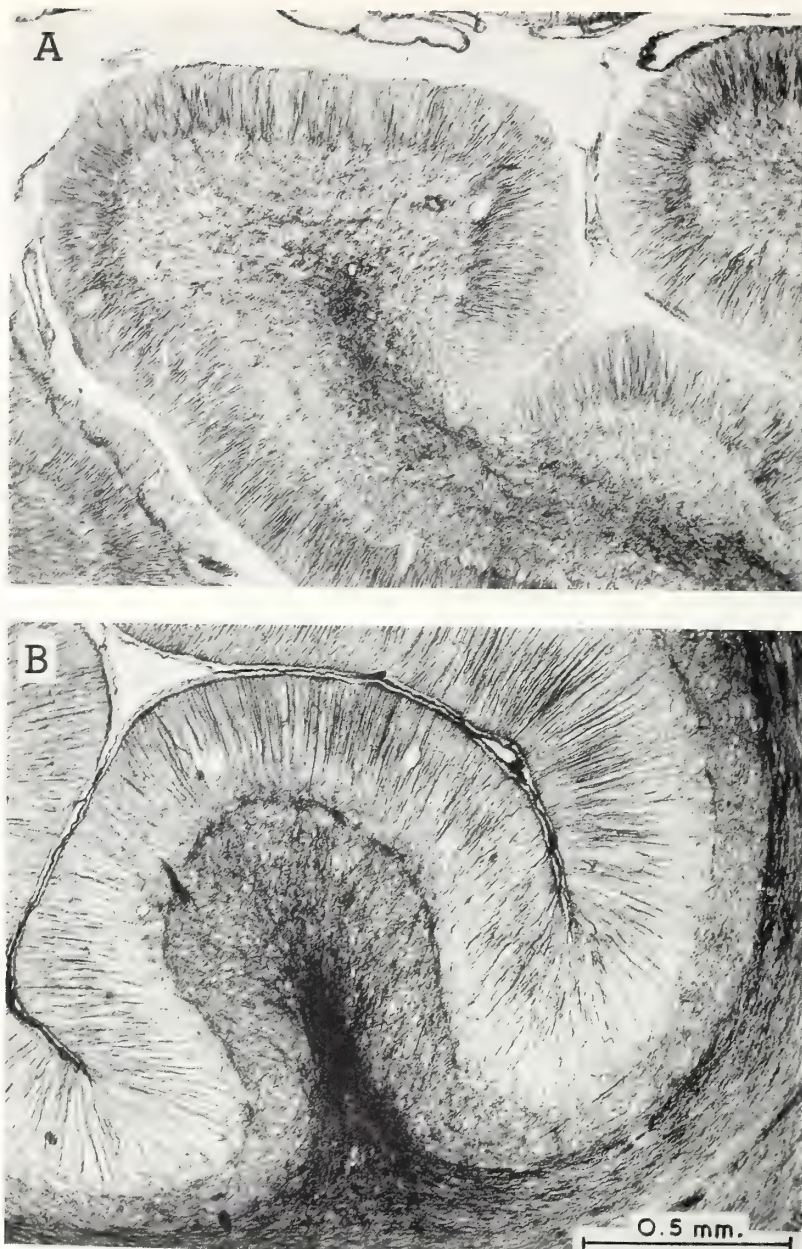


FIGURE 3.—Very dense fibrous gliosis in cerebellar cortex and white matter.
A, Kuru; B, Scrapie. (Holzer stain.)

also gave a positive reaction for neutral fat (fig. 5B). The entire white matter contained a dense network of glial fibers. The dentate nucleus was, on the whole, not very severely affected, showing neuronal vacuolation and neuronophagia in only a few cases. As in kuru many of the brainstem nuclei with afferent connexions to the cerebellum were degenerated and showed a dense fibrous gliosis (figs. 6B, 7B). In some of the less severe cases numerous vacuolated neurones were seen in the same nuclei, a phenomenon which we have interpreted as an atypical retrograde reaction of

the nerve cell to damage or degeneration of its axonal termination within the cerebellum. The middle cerebellar peduncles often contained quantities of neutral fat, while the inferior peduncles remained relatively normal. There was generalized astrocytic hypertrophy in the pyramidal tracts and in the basal ganglia which, however, showed no other pathological changes in any of our cases. (Unlike the findings of Bertrand, Carré, and Lucam (5) who reported selective vacuolation of anterior horn cells.)

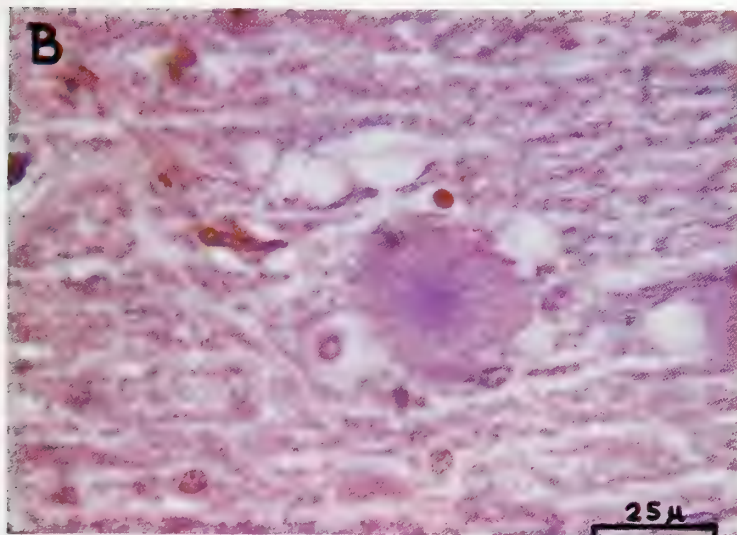
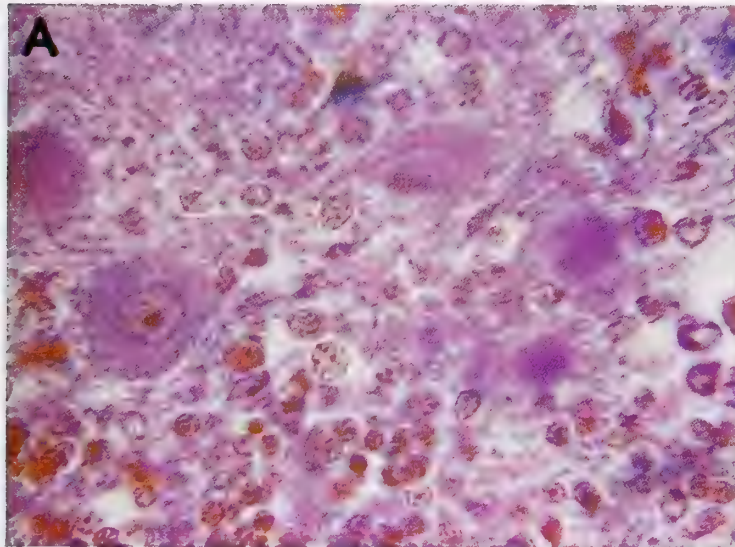


FIGURE 4. PAS-positive plaques within the granular and molecular layers of the cerebellum. These plaques are only faintly argentophilic and in this way differ from senile plaques.

A Kuru; B Scrapie (Periodic acid Schiff reaction).

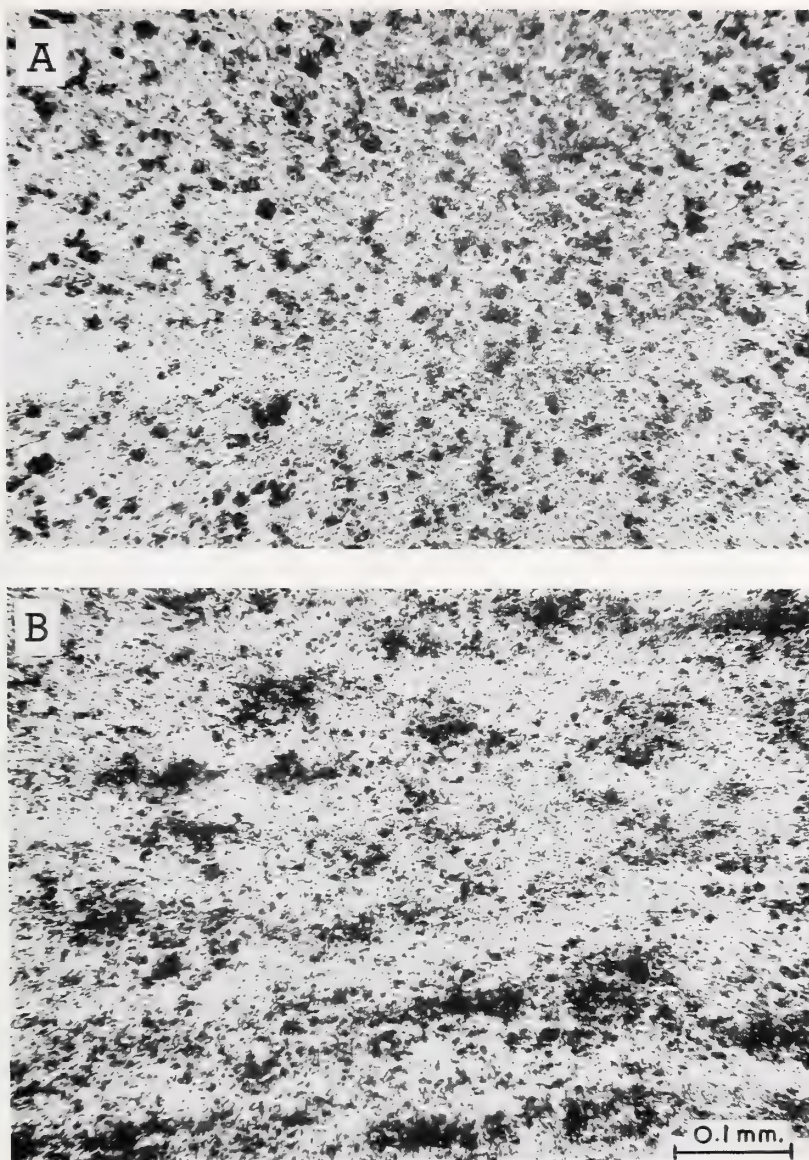


FIGURE 5.—Deposits of neutral fat in cerebellar white matter. A, Kuru; B, Scrapie. (Marchi stain.)

In scrapie the hypothalamo-neurohypophysial system showed an unequivocal selective degeneration. There was severe to moderate nerve cell loss in the supraoptic and paraventricular nuclei with a fibrous gliosis confined to the territory of these nuclei. In the median eminence and pituitary stalk there was often a large increase in neurosecretory substance as compared with normal controls while the neural lobe of the pituitary gland appeared comparatively empty. This picture fits in well with that seen in our cases of pituitary stalk section (6) and can be interpreted as an attempt at regeneration in the neurosecretory system.

DISCUSSION

When Hadlow (7) first drew attention to the similarity between the pathological changes in kuru and in scrapie he based his analogy mainly on the widespread "nonspecific" changes and the neuronal vacuolation common to both diseases. Whilst we agree with previous investigators that, in both kuru and scrapie, widespread "nonspecific" changes do occur in the central nervous system, comparable changes are also known to develop in the brain as a result of a wide range of noxious stimuli. We were therefore more impressed by the striking impact of the disease process upon certain specific neuronal sys-

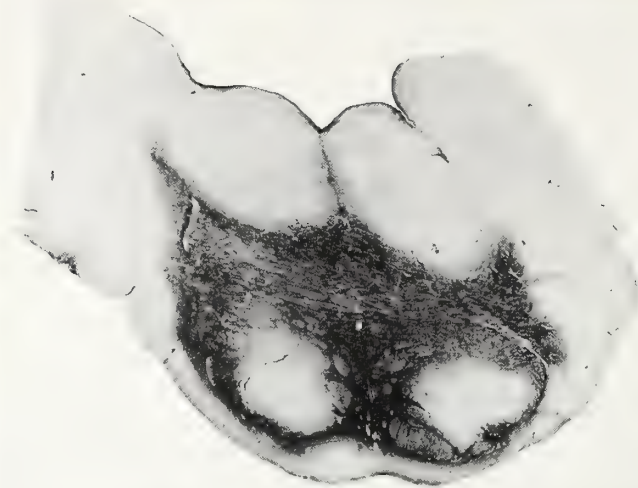
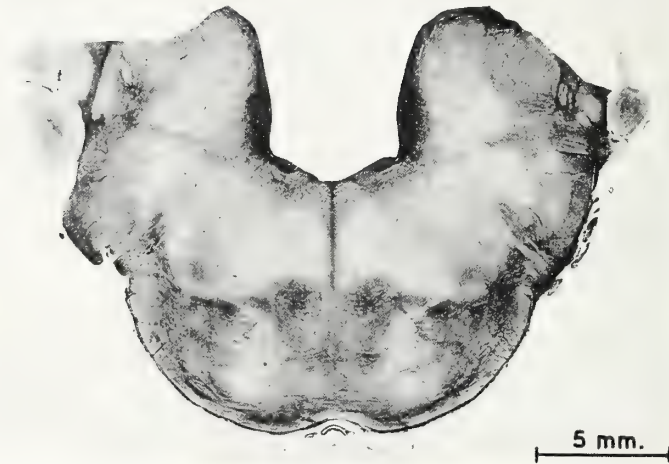
A**B**

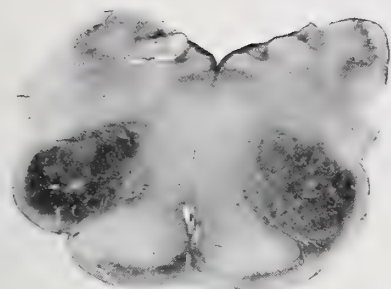
FIGURE 6.—Dense fibrous gliosis outlining the pontine nuclei and their decussating fibers (note that the pyramidal tracts are not gliosed). A, Kuru; B, Scrapie. (Holzer stain.)

tems, especially by the cerebellar system and in particular its flocculo-nodular lobe, degeneration of which leads to those clinical signs, ataxia and tremor, so prominent in both diseases. Severe degeneration of the flocculo-nodular lobe was noted by Fowler and Robertson (8) who related some of the early clinical signs to the lesions in this region.

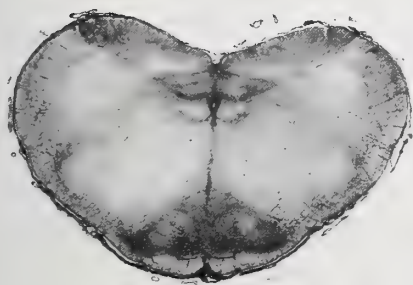
In the course of our studies it became increasingly clear that any comparison between kuru and scrapie should not stop at the "nonspecific" changes but should include the selective degeneration of the cerebellar system and possibly of the hypothalamo-neurohypo-

physial system. (Whether the changes found in the latter system in the case of kuru are to be regarded as significant will depend upon the study of adequate control material.) Such a concept would bring both kuru and scrapie within the realm of "system degenerations." In fact we were much impressed by the close resemblance of both diseases to some of the well recognised system degenerations in man, in particular those taking a relatively rapid clinical course, e.g., subacute cerebellar degeneration associated with carcinoma, cerebello-olivary degeneration associated with mental deterioration (Akelaits (9)) and olivo-ponto-

A



B



5 mm.

FIGURE 7.—Dense fibrous gliosis of the inferior olives and the lateral cuneate nuclei. A, Kuru; B, Scrapie. (Holzer stain.)

cerebellar degeneration associated with pulmonary tuberculosis (Rosenhagen (10)). In all these conditions there is the same loss of Purkinje and granule cells, the same fibrous gliosis in cerebellar cortex and white matter, the same lack of obvious demyelination (although myelin breakdown products are abundant (fig. 8 A to C)) and the same degeneration in brainstem nuclei projecting to the cerebellum (fig. 9). Lastly we have seen the same “nonspecific” astrocytic hypertrophy in some of our cases of carcinomatous cerebellar degeneration though never any neuronal vacuolation. We therefore feel that both kuru and scrapie are examples of a degenerative disease of the cerebellar system which in certain cases may be obscured by the very widespread “nonspecific” changes. The fact that scrapie can be transmitted through an “unorthodox agent” to experimental animals and the fact that there is such a remarkable similarity in the pathology of scrapie as compared with kuru and other cerebellar system degenerations makes one wonder whether some “agent” may also be implicated in the aetiology of such other conditions.

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DISCUSSION

JOHNSON: When I was in the Highlands I visited Dr. Hornabrook and saw his cases of kuru. The only thing I had previously seen resembling kuru was one particular case of carcinomatous degeneration of the cerebellum. The pathological findings presented thus interest me particularly. Carcinomatous degeneration of the cerebellum is a disease of totally unknown etiology. It is not due to metastatic disease to the cerebellum. It is a subacute cerebellar degeneration occurring in conjunction with carcinoma as a distant complication, most commonly with carcinoma of the ovary, lung, and breast. It has been speculated that it is a nutritional disease, but, in fact, the degenerations of the cerebellum in malnourished alcoholic patients and in patients with

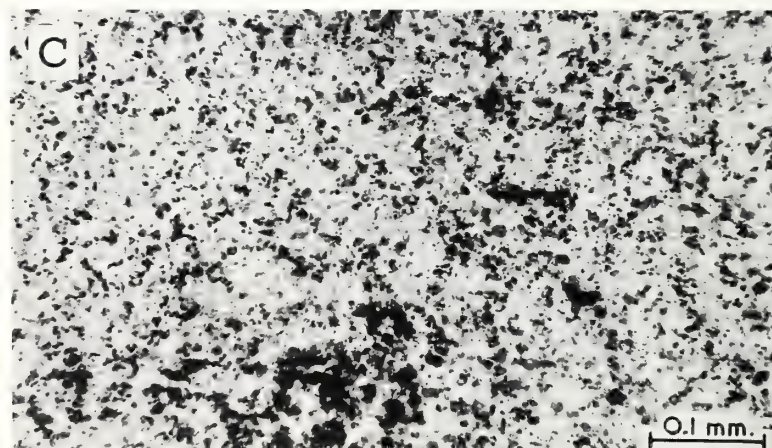
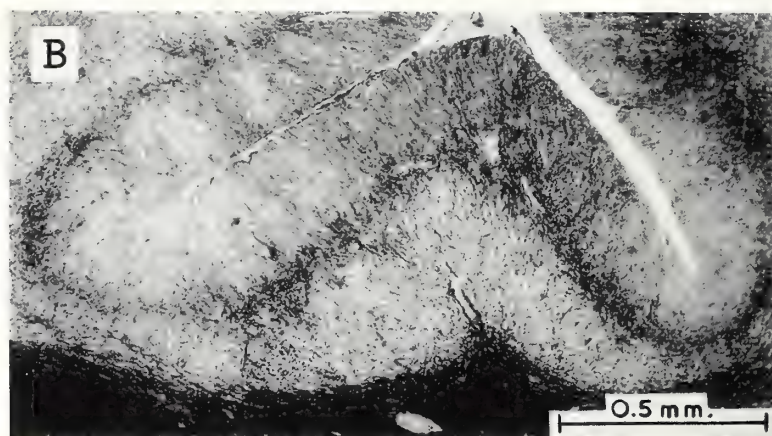
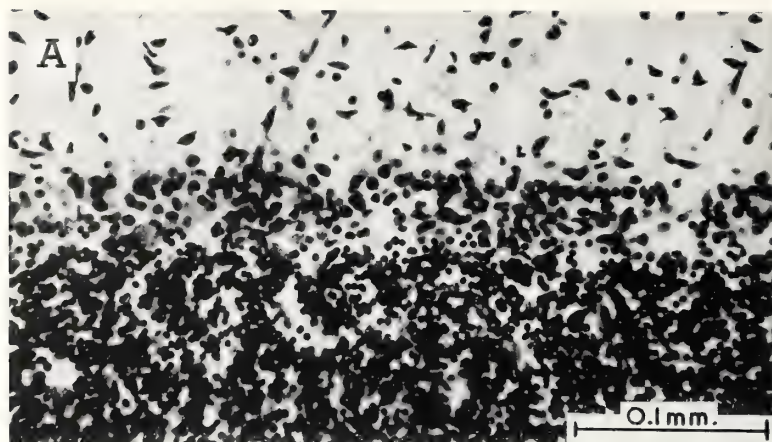


FIGURE 8.—Carcinomatous cerebellar degeneration. A. Cerebellar cortex showing severe loss of Purkinje cells and some rarefaction in the granular layer. B. Dense fibrous gliosis in cerebellar cortex and white matter. C. Deposits of neutral fat in cerebellar white matter.
(Nissl, Holzer, and Marchi stains.)



FIGURE 9.—Carcinomatous cerebellar degeneration. Dense fibrous gliosis of the inferior olives and the lateral cuneate nuclei. (Holzer stain.)

carcinoma are quite distinct clinically and pathologically. Furthermore, it does not occur with any regularity with the associated diseases; nor does it occur when the disease is at a certain degree of severity, for it sometimes may be the first symptom of carcinoma.

PATERSON: This entity being mentioned here is different from the degenerative changes seen with lymphoma and Hodgkin's disease?

JOHNSON: Progressive multifocal leucoencephalop-

athy. Yes, it is different pathologically and it occurs with a different spectrum of malignancy.

FIELD: There are also some other different forms of carcinomatous degeneration, of the cord, for example.

HARNER: An associated ALS syndrome with degeneration of both the lateral columns and the anterior horn cells has very recently been reported by Norris and Engel; and there is also a carcinomatous myopathy which has been well recorded by Wilkinson (Henson, R. A., Russell, D. S., and Wilkinson, M., *Brain* 77: Part 1, 82-121, 1954), and others since. I wonder if anyone has examined muscle from these sheep or from the patients with kuru.

BECK: I do not know about the patients with kuru, but in sheep the muscles were examined by Bosanquet, Daniel and Parry, and they found myopathic changes. In a second series we had 28 sheep in which muscles were available and though they did not show the severe changes described in the first paper there were definite myopathic changes in 27 of them. Now, we may be able to link these abnormalities with the hypothalamic changes found in scrapie through some metabolic or hormonal disturbance.

JOHNSON: Have you seen hypothalamic changes in any of the carcinomatous degenerations of the cerebellum?

BECK: No, not so far.

Pathology of Kuru and Other Exotic Central Nervous System Syndromes in New Guinea¹

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I have had the privilege of studying the brains of several New Guinea patients manifesting severe neurologic disorders (1). Three of them from the Eastern Highlands were clinically diagnosed as suffering from kuru; a fourth from the Southern Highlands had a different semiotic pattern, while a fifth, also from the Southern Highlands, succumbed to an acute ascending paralysis believed to be of infectious origin. Since the clinical picture of kuru is now well known to this audience, it will not be described, but it should be recalled that many of the presenting manifestations are suggestive of cerebellar dysfunction.

All of the brains were received unsectioned in 10-percent formalin. Only in case 3 was a lesion visible grossly: sclerotic folia in the cerebellum. Sections were taken from numerous cortical and subcortical areas, generally symmetrical from the basal ganglia and thalamus. The spinal cord was received in only one case: that of the ascending paralysis. All sections were stained by the following methods: Klüver-Barrera (Luxol blue-cresyl violet), Bodian or Holmes, Gomori trichrome, Holzer, PAS, hematoxylin-eosin. Some representative sections were stained with Alcian blue, oil red O pyridin, Perls iron reaction, Congo red, Cajal gold sublimate, and Ziehl-Neelson for acid-fast pigment in nerve cells.

Two of the three kuru patients (cases 1 and 2) showed the characteristic plaques now associated with this disease; they were first described by Klatzo, Gajdusek, and Zigas (2) and Fowler and Robertson (3).

These plaques (fig. 1A) were PAS-positive, stained blue with the Gomori trichrome method (using anilin blue), pale blue with Alcian blue, very pale pink with eosin, and faintly lavender with cresyl violet. Only in case 2 were they visible with the Bodian method (fig. 1B); the majority of the plaques remained unstained by silver techniques. They did not react for amyloid (Congo red method), and were pale mauve in the oil red O method counterstained with hematoxylin. In the Holzer technique they were unstained.

The plaques were widely distributed in the cerebellum in the granule and molecular layers, but were most numerous in the former region. They were not found in the medullary substance or in the cerebellar nuclei. They were distributed randomly, and not confined to any one area. For example they were observed in the flocculus (archicerebellum), anterior lobe (paleocerebellum), and posterior lobe (neocerebellum). They were also observed in the vermis. Plaques were rarely present in the thalamus and amygdala (case 2, fig. 1C) and cerebral cortex (cases 1 and 2). None were found in the brainstem.

Other changes in these two kuru cases were of a nonspecific nature. In the brain of case 1 (a 28-year-old woman) there were degenerative changes in the Purkinje cells with torpedo formation, and sponginess of the cortex and caudate nucleus. Fine red-stained lipoid granules, never extensive, surrounded glia nuclei in the granule and Bergmann's layers. In case 2 (a boy of 14 years), the plaques were exceptionally numerous, and torpedo formation was also prominent. The sponginess of the gray matter was striking, most marked in the cortex (fig. 2A), where there was loss of nerve cells and mild reactive gliosis (fig. 2B). The

¹ A detailed report of this work was published in the *Journal of Neuropathology and Experimental Neurology* (co-authors, D. C. Gajdusek and V. Zigas) 23: 486-507, July 1964. A full bibliography is included in that article.

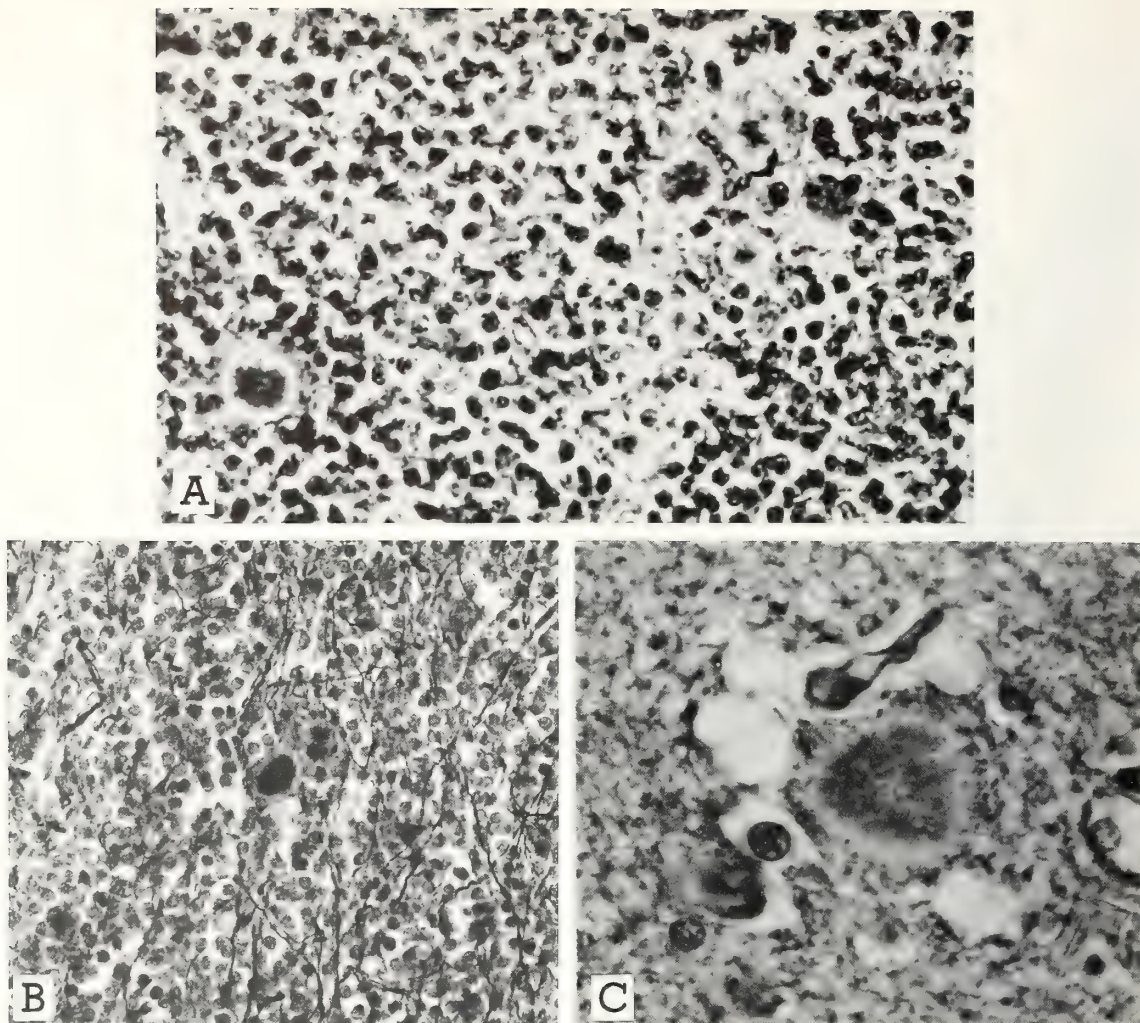


FIGURE 1.—*A. Case 1.* Plaques in granule layer of cerebellum. PAS method. $\times 350$
B. Case 2. Plaques in granule layer of cerebellum. Holmes method. $\times 300$
C. Case 2. Plaque in amygdala surrounded by "microcysts." Gomori trichrome stain. $\times 700$

basal ganglia and thalamus also showed this sponginess (fig. 2C); in the white matter there were small cystlike spaces where myelin sheaths and axis cylinders were thinned out. No vacuolated nerve cells were observed in any of the sections. Gliosis of the inferior olives was particularly intense in case 2.

The third case from the kuru district was a 23-year-old male. His tremors, gait disturbance, ataxia, dysarthria progressing to unintelligible speech, were not considered different from other kuru cases, but no plaques were demonstrated in any area of the brain. The anterior lobe of the cerebellum showed sclerotic atrophy with fibrillary gliosis in the molecular layer

and pronounced astroglial reaction in the granule layer. Occasional torpedoes had formed. There were symmetrical small heterotopias in the medullary substance. The pons showed central myelinosis of the base with reactive gliosis. Focal astrocyte hyperplasia was present in each caudate nucleus and putamen. In the upper cervical cord there was gliosis of the ventral horns, central gray matter, and zones of Lissauer. (Lower levels of cord were not available.)

The fourth patient, who lived outside of the kuru area, manifested a tremor syndrome distinct from kuru, and later had severe involuntary movements of the head, trunk and all extremities, and ataxia. Mi-

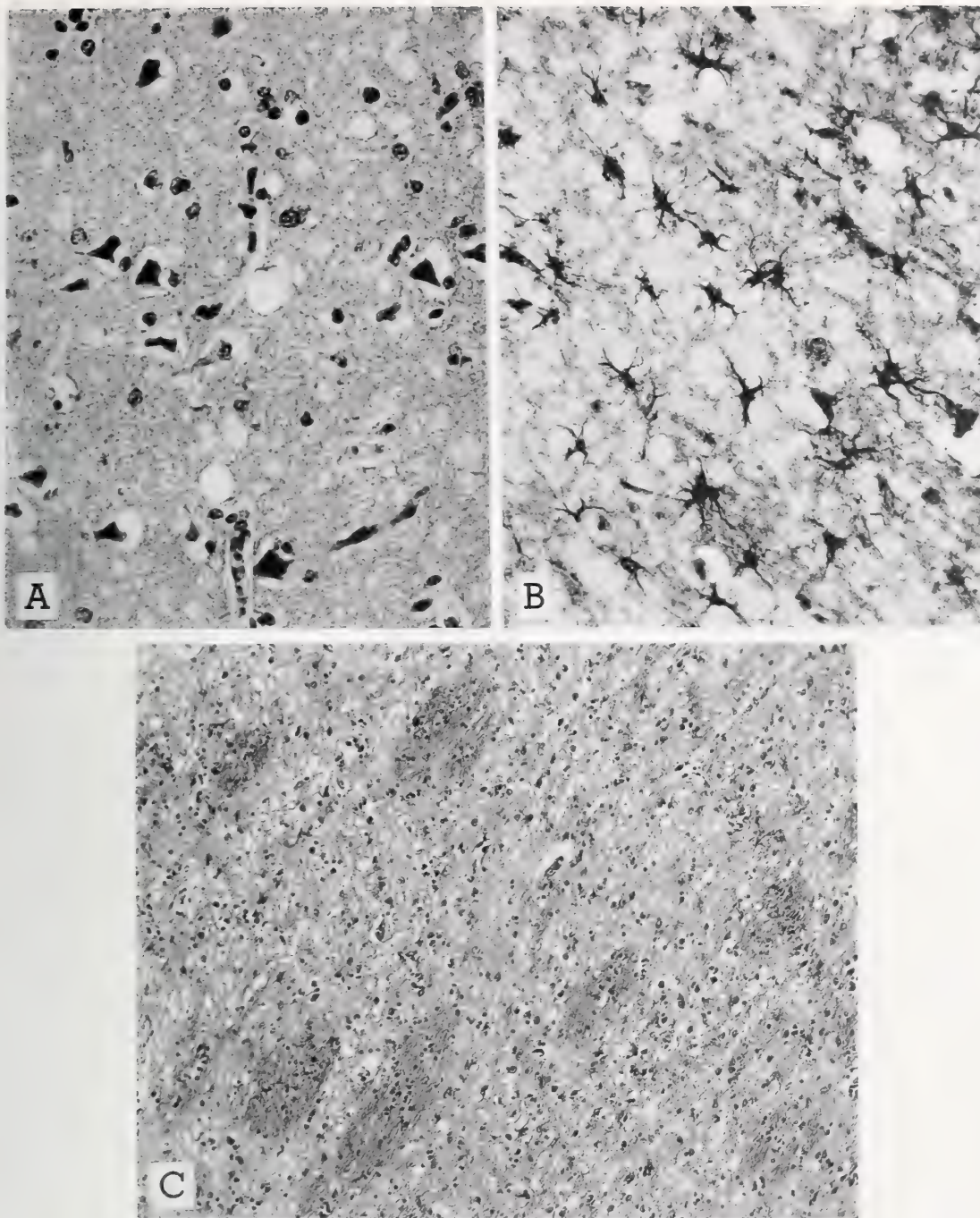


FIGURE 2.—*A. Case 2.* Spongy cortex and pyknotic nerve cells. Hematoxylin-eosin. $\times 300$
B. Case 2. Astrocytosis in spongy cortex. Cajal gold sublimate. $\times 250$
C. Case 2. Sponginess of ground substance in caudate nucleus, but not in myelinated bundles of nerve fibers. Gomori trichrome stain. $\times 92$

croscopically there was patchy ill-defined loss of myelin (figs. 3A, C), random in distribution; all foci showed reactive gliosis in the Holzer preparations (figs. 3B, D). The axis cylinders were not severely damaged in the demyelinated regions. Torpedoes were prominent in the cerebellum (fig. 4A). Many nerve cells showed central chromatolysis. In both gray and white matter hypertrophied microglia and astrocytes were present; there were a few glia nodules. Perivascular infiltrations with lymphocytes, particularly in the medulla, and extensive sponginess of the parenchyma, were additional features (fig. 4B). With the oil red O technique, orange-red globules of lipid material were demonstrated in the low medulla in the decussation of the pyramids, more on one side. There were also many lipid granules in the descending and transverse fibers of the pons, brachium pontis, and to a less extent, in the tegmentum. In the cortex lipid deposits were observed in the dystrophic white matter, generally engulfed in macrophages.

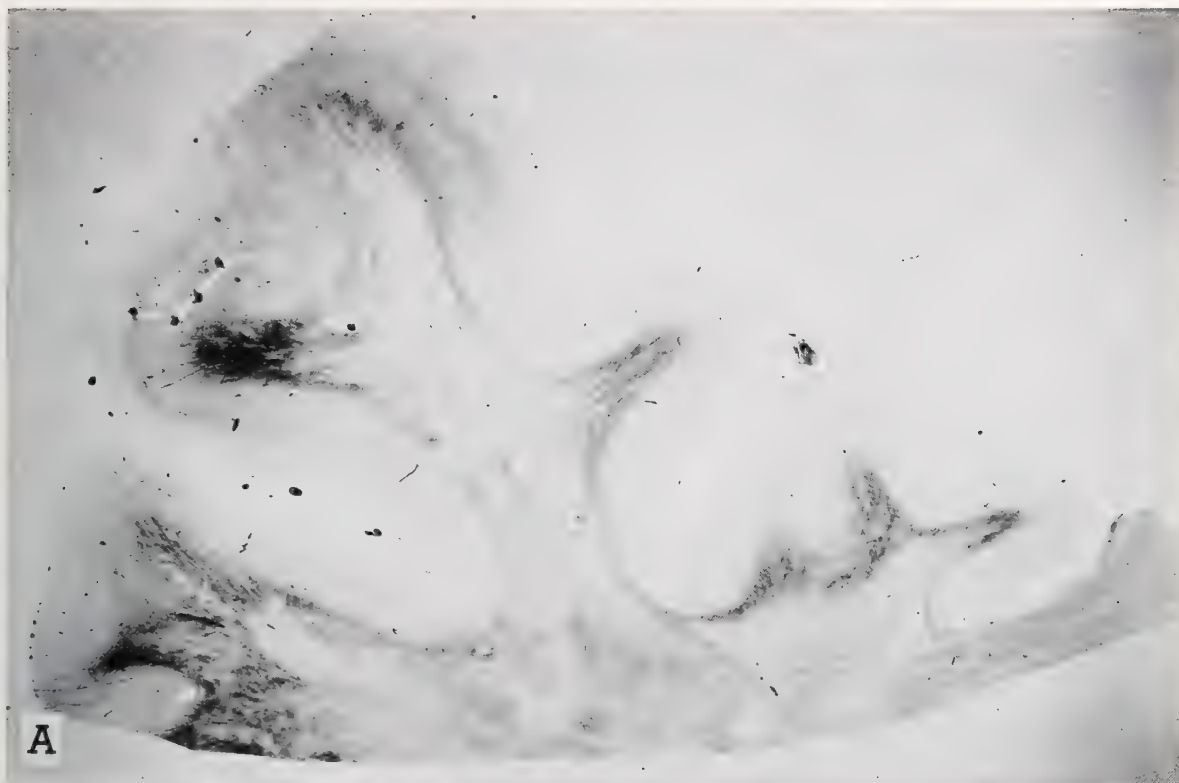
The fifth case, also from outside of the kuru district, developed a sudden rapidly progressive Landry's type of ascending paralysis. He died on the fourth day of his illness, following bulbar involvement. The lung sections showed a hemorrhagic pneumonitis. The brain was congested and edematous. Microscopically there was severe diffuse involvement of the gray and white matter of the brainstem by an inflammatory process. In keeping with the history the changes were most intense in the spinal cord. Axonal degeneration was conspicuous in the ventral horns where very few motoneurons remained (fig. 5A). Lymphocytes and plasma cells predominated among the defense reaction cells, but in the medulla oblongata and higher brain stem polymorphonuclear leucocytes were more abundant, indicating a more acute phase. Myelin had disappeared in small inflammatory foci. Small nodules of mixed glia cells and lymphocytes were prominent. The inflammatory reaction was present in the cerebellum (fig. 5B), tectum, tegmentum and periventricular regions of the thalamus, but the basal ganglia and cerebral cortex were not affected.

Since the publication of these five cases a brain from another New Guinea patient was studied by similar methods. He was a middle aged man who had been diagnosed as suffering from encephalitis; his illness was acute and he died on the fourth day after the onset of symptoms. Other pertinent data were not available. Viral studies were carried out by Dr. R. L. Doherty of the Queensland Institute of Medical Research, but no specific agent was identified.

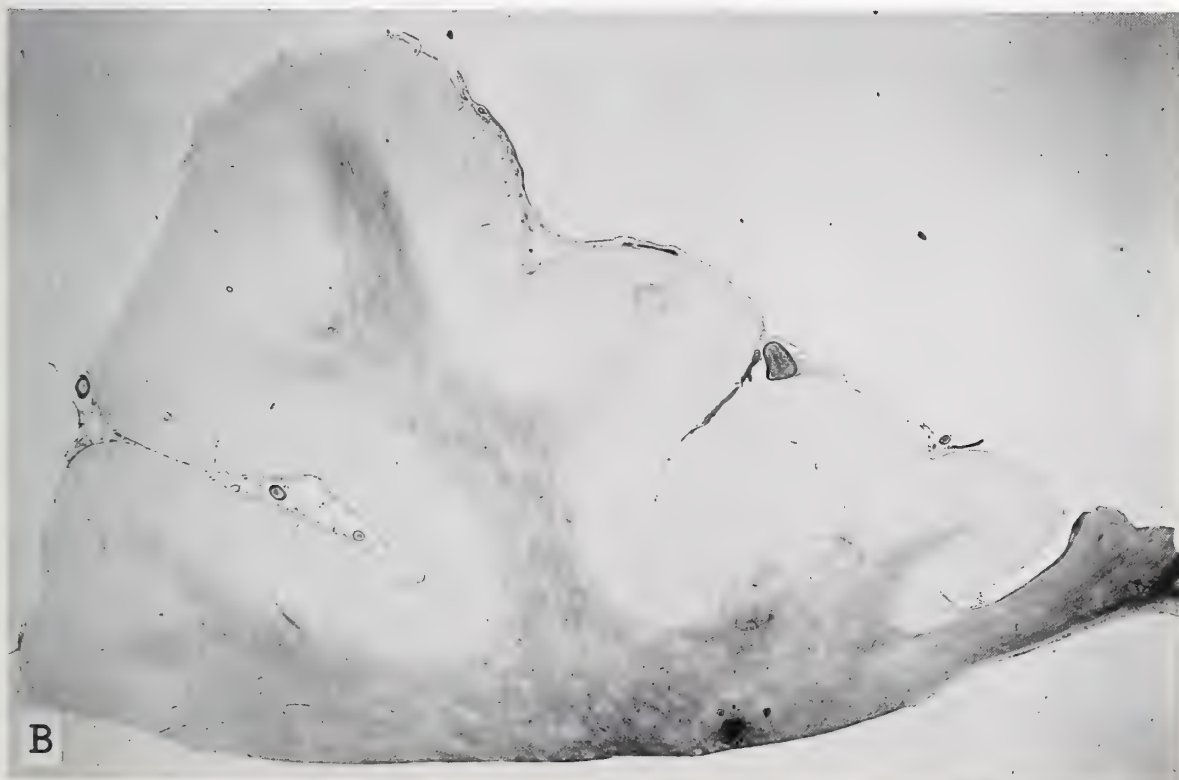
The brain showed congestion and edema on gross inspection. The folia of the left cerebellar hemisphere were slightly smaller and paler than those on the right. Microscopically the most striking features were the numerous small areas of necrosis not associated with vascular occlusions. These were observed in the right frontal operculum, left amygdaloid nucleus, right and left caudate nuclei, and other basal ganglia areas, septal nuclei, basal olfactory region, lateral nucleus of thalamus, dorsal thalamus, tegmentum of pons, mid-brain and right brachium pontis. These foci were characterized by capillary proliferation, congestion, slight perivascular infiltrations, gitter cells (fig. 6A) and hypertrophied microglia. The parenchyma at the sites of the lesions was spongy (fig. 6B), and showed loss of neural elements; degenerated axis cylinders were found in adjacent tissues. Large pale-stained astrocyte nuclei were observed in or near the lesions (fig. 7A). The proliferation and hypertrophy of microglia were prominent in almost all sections, but particularly in the basal ganglia and cerebellum. The cellular defense reaction was most intense in the interpeduncular fossa, roof and walls of the fourth ventricle. Lymphocytes predominated in the cellular exudate of parenchyma and meninges. There was decrease of myelin in several subcortical areas, and small definite foci of demyelination in the tegmentum of the pons and decussation of the brachium conjunctivum. The cerebellum showed focal loss of Purkinje and granule cells (fig. 7B); no torpedoes were observed. There was proliferation of Bergmann's glia and of the microglia in the medullary substance and molecular layer (fig. 7B). In this latter region they often appeared as "glial shrubbery" described by Greenfield (4) as occurring in viral infections, particularly in Japanese B encephalitis. All of the described changes are characteristic of viral infections, although generally such diseases are accompanied by a more severe and acute cellular defense reaction (4).

Since this symposium is on viral infections, it should be stressed that in only two of the New Guinea cases of the group which I personally studied were the histologic changes definitely compatible with virus diseases (cases 5 and 6). The disease was rapidly fulminating in both patients, death occurring on the fourth day of the illness in each.

In known viral diseases where recovery from the acute process occurs, and the patients may survive for prolonged periods, cellular defense reaction and even microglial proliferation may be absent at the time of death, but loss of neuronal elements and dense astrocytosis will mark the site of the attack of the viral



A



B

FIGURE 3.—A. Case 4. Subcortical loss of myelin, frontal lobe. Luxol blue.
B. Case 4. Reactive gliosis in area of demyelination. Holzer stain.

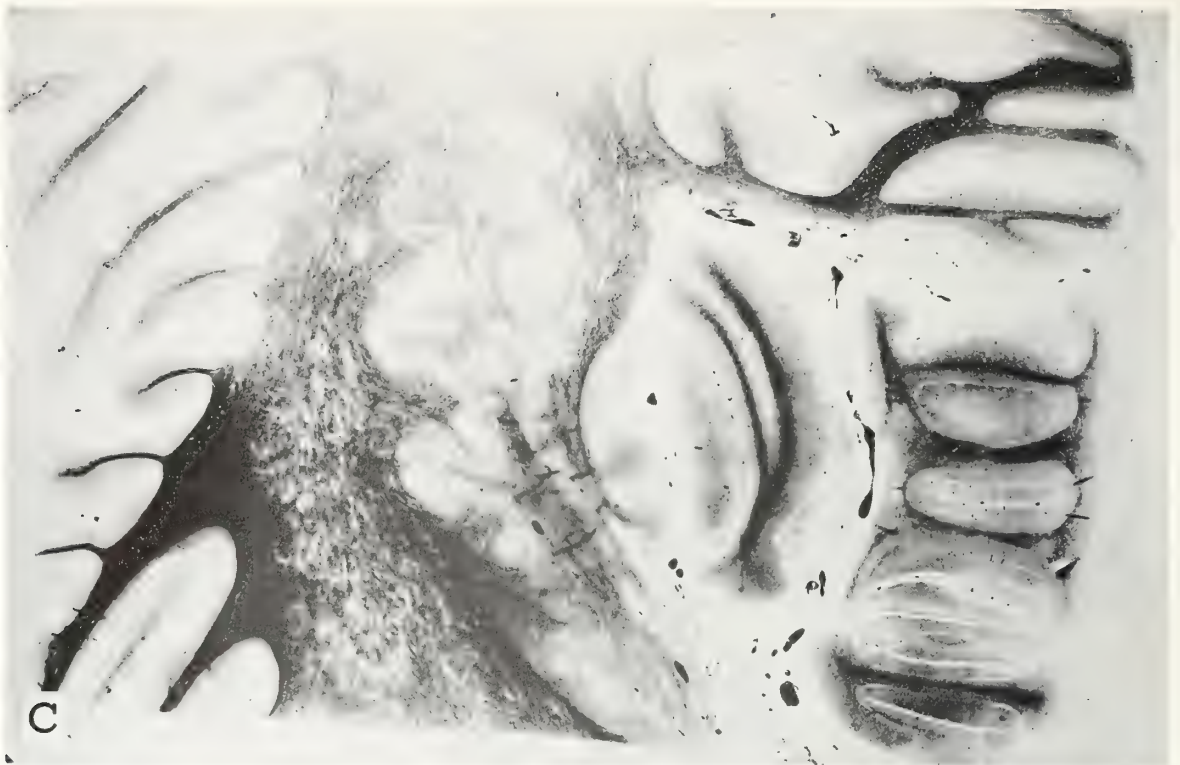


FIGURE 3. *C. Case 4.* Myelin loss in cerebellum. Klüver-Barrera.
D. Case 4. Reactive gliosis particularly in hilus of dentate nucleus. Holzer stain.

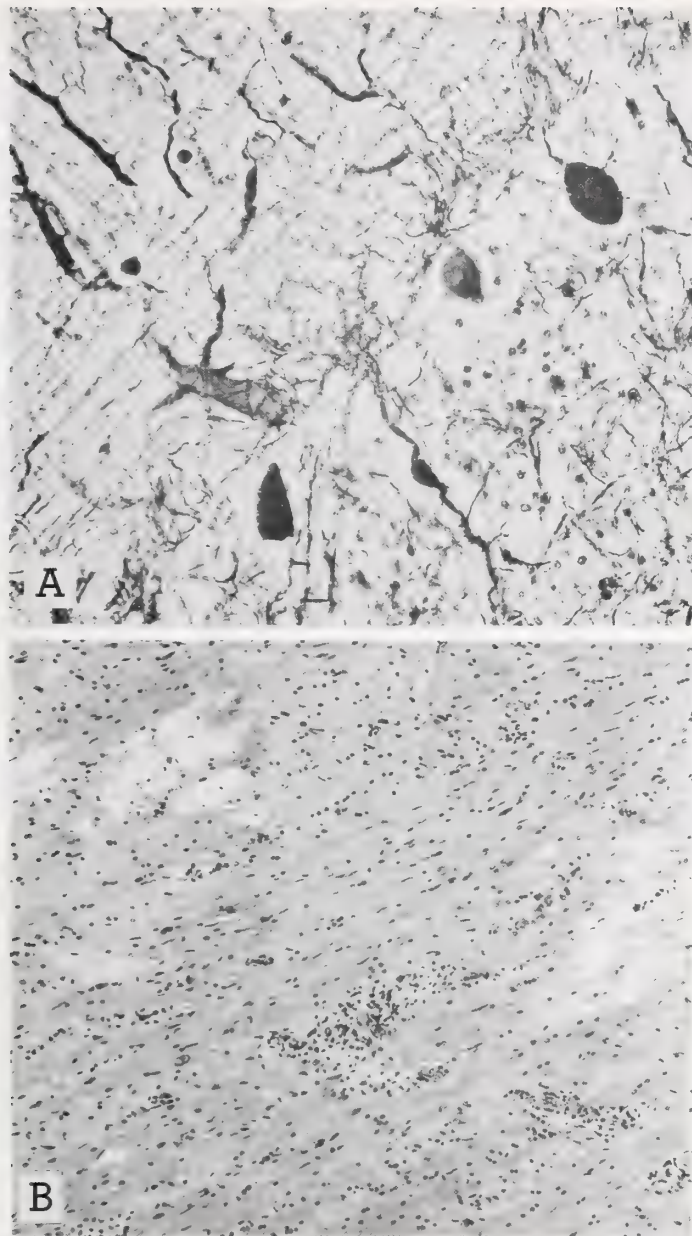


FIGURE 4.—*A. Case 4.* Purkinje cell degeneration with torpedo formation. Decrease of granule cells. Bodian stain. $\times 300$
B. Case 4. Sponginess and inflammatory focus, globus pallidus. Hematoxylin-eosin. $\times 93$

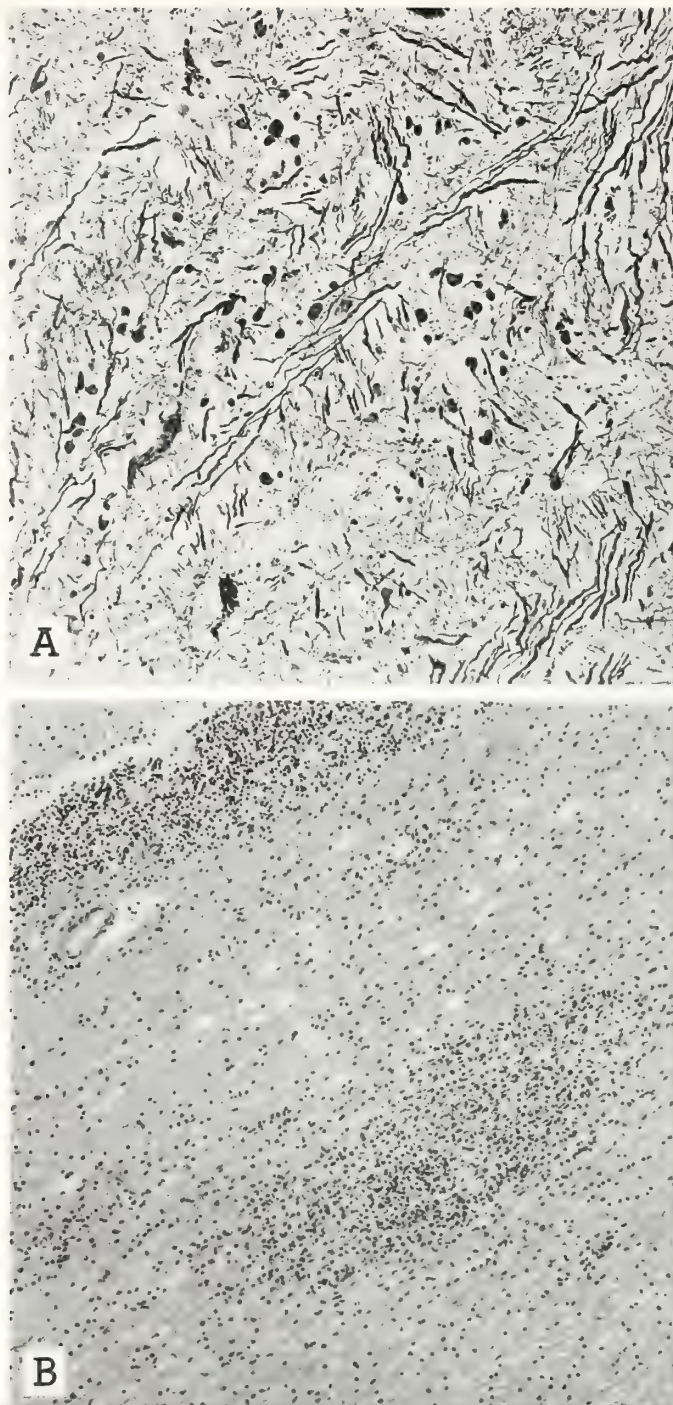


FIGURE 5.—*A.* *Case 5.* Axonal degeneration in ventral horn of spinal cord; loss of motoneurons. Bodian stain. $\times 92$
B. *Case 5.* Focus of polymorphonuclear cells, cerebellum. Hematoxylin-eosin. $\times 92$

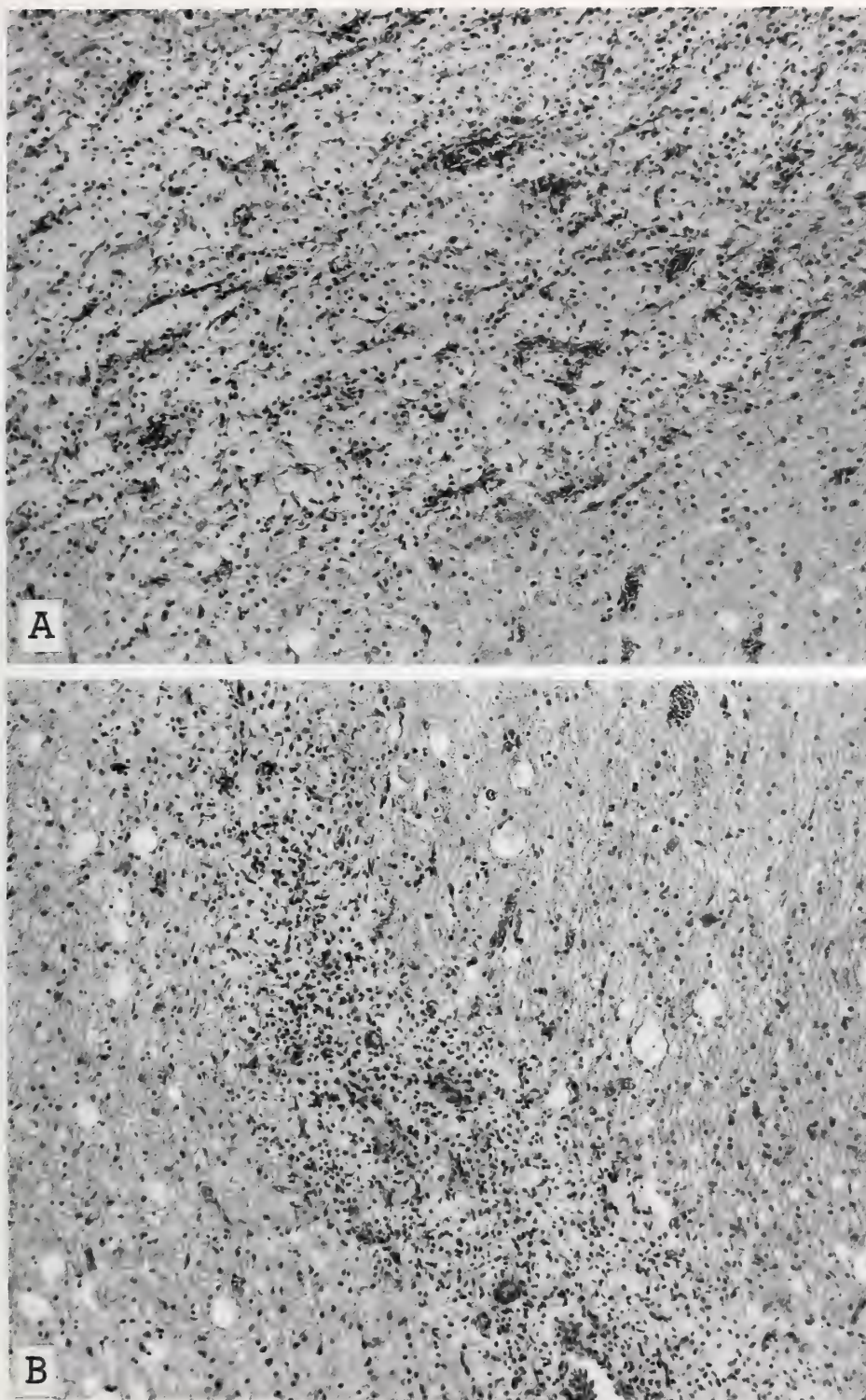


FIGURE 6.—A. *Case 6*. Midbrain. Focus of gitter cells in tegmentum; capillary proliferation. Hematoxylin-eosin. $\times 140$

B. *Case 6*. Focus of inflammatory and glial cells in tegmentum of midbrain with adjacent spongy parenchyma. Hematoxylin-eosin. $\times 140$

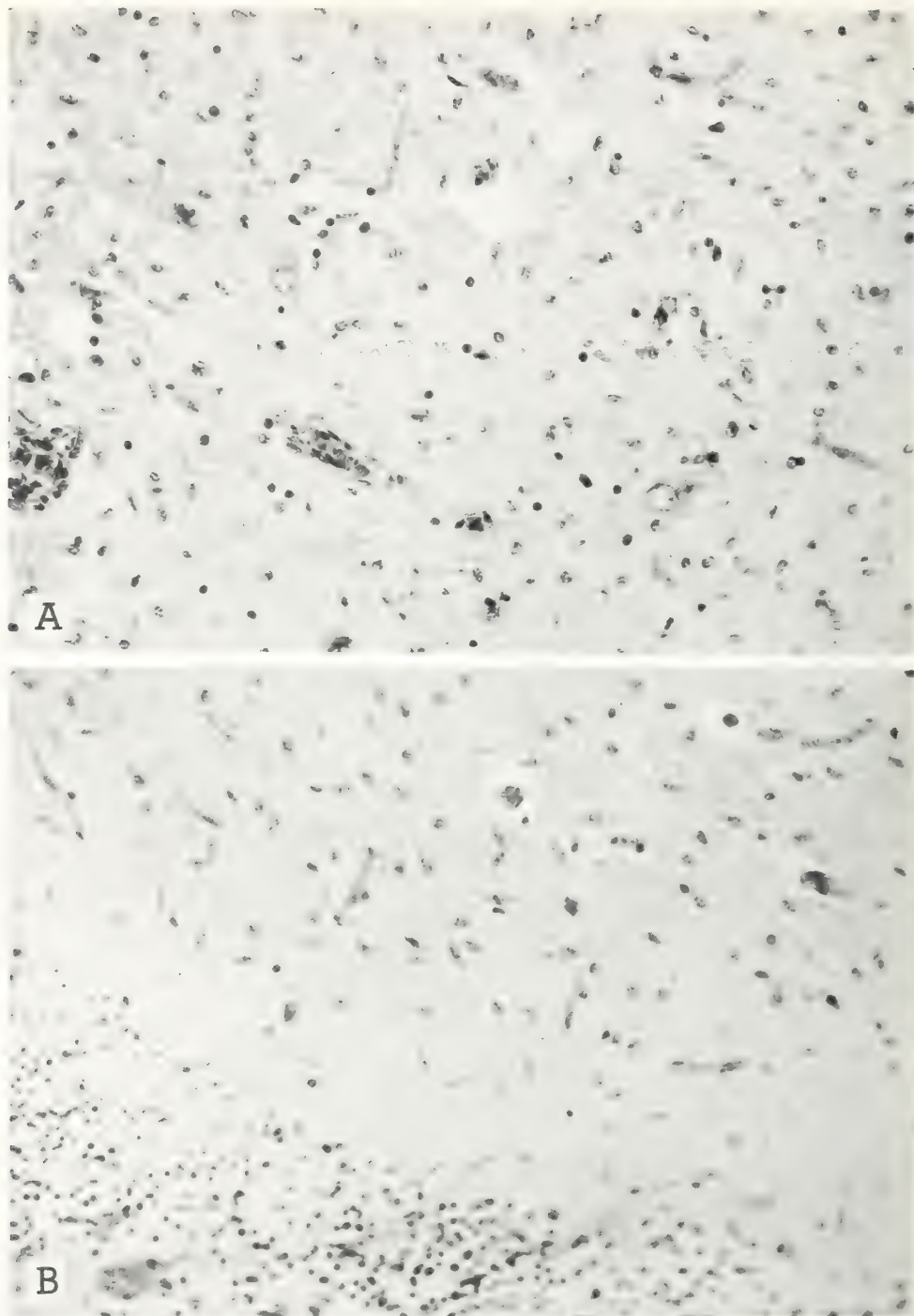


FIGURE 7.—*A. Case 6.* Recent necrotic focus in globus pallidus; hypertrophy of microglia and large pale astrocytes. Hematoxylin-eosin. $\times 300$
B. Case 6. Cerebellum. Loss of Purkinje and granule cells; proliferation of microglia in molecular layer. Hematoxylin-eosin. $\times 300$

agent (for example, anterior horns in anterior poliomyelitis, or substantia nigra in chronic epidemic encephalitis). These histologic changes are quite different from the acute phases of viral disease such as described in this report, and which are generally more diffuse in distribution. The clinically diagnosed cases of kuru showed an entirely different picture, which had no pathologic resemblance with the changes found in the brain in diseases attributed to, and proven to be, of viral origin. Likewise Klatzo, Gajdusek, and Zigas stated in their study "that the pathologic findings in kuru do not resemble closely any known picture of viral infection".

The various types of inclusion encephalitis, which are most likely of viral origin, show specific histologic changes and will not be discussed here since they are not pertinent.

Although the cerebellum is the site of predilection for conspicuous tissue alterations, lesions were present in other areas of the brain and I do not believe that these kuru cases can be classified as "system" degenerations. The "spiked balls" were random in distribution, and in our cases were found in the granule and molecular layers. Their absence in known cerebellar connections and cerebellar nuclei is significant, particularly if one considers them to be products of neuro-axonal degeneration, as some investigators who have found seemingly similar structures in other pathologic conditions of the central nervous system believe (5, 6, 7, 8). I myself remain unconvinced that the kuru plaques arise from degenerated axis cylinders, partly because of their limited distribution and the fact that in only one instance were they demonstrated with silver techniques.

Neurologic disorders involving the cerebellum vary considerably both in clinical and pathological manifestations. This has been pointed out at various times (9, 10, 11, 12, 13). In fact it has also been noted that it is sometimes difficult to determine from clinical studies alone, just which part of the cerebellar pathways should be implicated. Nevertheless there are certain disorders which are clearly cerebellar system diseases, although there is often considerable variation between the degree of involvement of the various elements of the system. The acquired or late cortical cerebellar atrophy, also known as parenchymatous cerebellar degeneration, is a relatively common finding in neuropathologic investigations. As Greenfield (10) pointed out, this condition is not a systemic degeneration. Although in kuru the Purkinje layer is severely affected as in cortical cerebellar atrophy, the widespread involvement of other cerebral areas, particularly

the sponginess of both gray and white matter, precludes even classifying kuru as a diffuse cerebellar disease. Klatzo, Gajdusek, and Zigas (2) in their earlier detailed study likewise came to the conclusion that kuru was not a systemic disease. Our case 3 with sclerotic atrophy of the cerebellum and no plaques can more surely be grouped with the cerebellar disorders from the pathologic standpoint. The changes were most severe in the anterior lobe, which receives spino-cerebellar fibers. No change was observed in the spino-cerebellar tracts in the cervical level (lower levels were not available) or in the medulla. Although there was central loss of myelin in the basis pontis, with reactive gliosis indicating this was not recent, each brachium pontis was well myelinated and free from glial reaction.

Case 4 with its foci of myelin loss, gliosis, glia nodules, hypertrophied astroglia, microglial proliferation, and perivascular infiltrations, appears to be in a class by itself. The etiology is obscure: it was not recognized as an infectious process clinically, but was diagnosed as a neurologic disorder of probable degenerative type. Some pathologic features nevertheless suggest a viral origin: perivascular defense reaction and response of astrocytes and microglia. The patient's illness lasted 10 months.

Finally the spongy changes in the parenchyma which were so prominent in the two cases with "spiked balls" and in case 4, and which were described by Klatzo et al., present a problem. As stated above the widespread distribution of these tissue spaces: cerebral cortex, gray matter of the basal ganglia (not in the fiber bundles), white matter of the cerebellum, mitigates against a classification of a system degeneration. Spongy encephalopathy has been described by Nevin, McMenemy, Behrman, and Jones (14), by Heidenhain and others among the degenerative diseases of the central nervous system. It is also a prominent feature of scrapie (15, 16).

Since scrapie has sometimes been compared with kuru, a few words are appropriate, although I have never personally studied a case. Hadlow (17), who was the first to make this comparison, admitted that all of the histologic alterations observed in both diseases are of a nonspecific nature, and this point should certainly be stressed at this symposium. Many of the investigators appear to have demonstrated the viral nature of scrapie from their experimental studies, and yet the hereditary factor has not been disproven. Parry's recent work shows the importance of the genetic factor. The detailed report of Beck, Daniel and Parry (18) pointing out the symmetry of the location of the lesions also is more indicative of a degenerative process

or an abiotrophy, rather than a viral disease, which would tend to produce randomly distributed lesions or attack a specific area. In this connection it is of interest that Parker and Kernohan (19) in 1933 in a report on parenchymatous cortical cerebellar atrophy suggested that the condition might be due to a virus with a special affinity for Purkinje cells. This has not received much attention in the literature, but in view of the discussions at this symposium merits attention.

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DISCUSSION

GAJDUSEK: Perhaps I should add a comment on the Western syndrome, of which we have now seen several cases. It occurs in an area over one hundred miles west of the kuru area. It is a violent tremor disorder restricted mostly to the upper extremities, with little ataxia, and no case among those we have seen had direct contact with any other, although some have a history of the disorder occurring in previous generations. The fact of finally getting the brain from one case and having Miss Neumann find it quite different in pathology from kuru is most important. We have no hypotheses about this disease and no clue to its etiology. It is of passing interest that in the same area where it occurs there is another chronic or subacute neurological disorder which is certainly infectious. Seasonally, on several occasions, in the Western Wabag area, an acute fasciculation syndrome has occurred which the natives describe as "worms and snakes" under the skin, and the manifestation of this can be seen in their near-naked bodies from far off. The fasciculations are coarser than in motor neuron disease and occur over vast areas of the body; when they involve the intercostal muscles and diaphragm the patient may die within the first week or two of the onset. There is no fever. This disease has occurred in many subjects at one time in a given village, or in adjacent villages where it had not been previously seen. I

have arrived months after it occurred, when many individuals who had it still demonstrated muscle masses which continued to fasciculate. We have followed a few that have continued to fasciculate in certain discrete muscles for many years; one male patient in particular continues to demonstrate year after year "the worms under his skin," as he calls it, on his right thigh. The rest of his body has ceased to show these fasciculations. The epidemiology of the disease clearly points to something infectious. The phenomenon that the fasciculations remain in muscles for months and years, in localized areas,

is an interesting neurological finding. It is another of these oddities that we have stumbled on in looking for kuru. In the New Guinea region populations with unusually high incidence of motor neuron disease are also found and in several regions, particularly the Usurufa, Auyana, Kanite and North Fore region which overlaps the northern part of the kuru region, there is an unusual accumulation of congenital tremors and also of atypical acquired parkinsonism-like syndromes. Thus there remain here many interesting problems for further investigation by neuropathologists.

Slow and Latent Virus Infections of Man

Chairman

E. J. FIELD

Chronic Sequelae of Tick-Borne Encephalitis and Vilyuisk Encephalitis

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INTRODUCTION

During the years following the demonstration of slow viruses in sheep capable of causing progressive lesions of the central nervous system (CNS), attention has naturally focused on the possible viral etiology of progressive diseases of the CNS in man. Soviet scientists for many years have believed in the viral etiology of chronic CNS disease, not only as the sequelae of acute infection but as the result of persistence of virus in nervous tissue. Other participants in this symposium have mentioned the strain of rabies virus which Professor Shubladze has proposed as the cause of multiple sclerosis, and the agent which Professor Zil'ber has suggested as the possible cause of amyotrophic lateral sclerosis.

I shall attempt a brief review of Soviet work concerning the chronic sequelae of tick-borne encephalitis and a description of a little known entity, Vilyuisk encephalomyelitis, which occurs in a small focus in northeastern Siberia. The data to be presented are, in part, a review of Soviet literature and, in part, information acquired in discussion with Soviet scientists during several visits to the U.S.S.R.

Chronic Sequelae of Tick-Borne Encephalitis

Soviet scientists began a systematic study of tick-borne encephalitides in 1937. In that year, Zil'ber led a Soviet scientific mission to eastern Siberia to investigate reports of numerous cases of encephalitis. Included in this group were many of the prominent Soviet virologists of today: Chumakov, Levkovich, Shubladze, and Soloviev. During the course of this study, the virus of tick-borne encephalitis (TE), was isolated and the tick vector *Ixodes persulcatus* was identified (1, 2).

Pavlovsky, in the following year, led an expedition to eastern Siberia with Smorodintsev as virologist. At this time, Pavlovsky developed his concept of the natural foci of infection and Smorodintsev produced the first mouse brain vaccine against tick-borne encephalitis.

Neurologists in the Soviet Union believe that chronic progressive disease of the CNS can follow infections with TE virus. Mental disturbances, sometimes of a progressive nature, are considered the most common sequelae of infection but such diseases as parkinsonism, amyotrophic lateral sclerosis, and multiple sclerosis are also thought, by some neurologists, to follow TE (2). A common sequela of TE is weakness and atrophy of muscles of the shoulder girdle; this, too, is believed to progress in some cases (2). Another clinical syndrome thought to occur after TE virus infection is epilepsy partialis continuo. In the U.S.S.R., this focal convulsive disorder is referred to as Kozhevnikov's epilepsy after the Russian neurologist who first described it in 1894 (3). It is more commonly seen as a sequela of TE than with any other clinical disorder and is said to be progressive, occasionally leading to death.

Investigators believe that the progressive nature of the disease is due to the persistence of the TE virus in the CNS, and in 1940 Chumakov isolated the TE virus from brain tissue removed surgically from a patient with Kozhevnikov's epilepsy and inoculated into adult mice (4). Another isolation of TE virus from a patient with chronic sequelae after infection is said to have been made by Schublin in Tomsk in 1956. These two observations remain unconfirmed. At varying intervals after the onset of acute TE, virus has been isolated from patients' blood. To date, no systematic attempt has been made to determine the

conditions under which virus can be isolated from patients with progressive disease or at autopsy.

Sequelae following TE are not common. Kozhevnikov's epilepsy is said to occur in only 1 or 2 percent of patients with diagnosed TE (2). Undoubtedly, severe sequelae can follow infection with this virus, but it is not fully established whether these conditions are progressive and related to the persistence of virus in the patients' tissue.

Vilyuisk Encephalomyelitis

Vilyuisk encephalomyelitis, a chronic neurologic disease possibly of viral etiology, is confined to a small area in Siberia inhabited by the Yakut Tribe. Explorers visiting the area north of Yakutsk between 1850 and 1900 described a strange debilitating neurologic illness among villagers along the Vilyui River. The first clear descriptions of this illness appeared in the Soviet literature in 1926 and 1930 (5).

As concern about the illness increased, Soviet medical teams went to the area each summer from 1954 to 1957 to conduct clinical, virological, and epidemiological investigations. During the 4 years of this study, fewer than 200 cases occurred. Of 290 cases recorded since 1952, 112 occurred in 50 families and, except for one case, all occurred among Yakuts (6). The age at onset was from 8 to 45 years, and the illness was more common among females (7).

Clinically, Vilyuisk encephalomyelitis occurs in two types, acute and chronic. When an acute phase occurs, it may begin suddenly or after a period of depression and it may last from several days to a month. The acute illness is characterized by mild influenza-like symptoms or by cranial nerve disturbances, extrapyramidal rigidity, tremor, and deep lethargy (8).

The chronic phase is sometimes characterized by increasing spasticity and dysfunction of the cranial nerves or extrapyramidal tracts. Personality changes and forgetfulness develop, progressing to severe dementia. The patients often survive in a demented, spastic state for 20 years or more (9). Five percent of cases develop symptoms of classical amyotrophic lateral sclerosis (8).

The pathology of the CNS is that of intense pan-encephalitis with inflammation and loss of neurons occurring at all levels. Hydrocephalus is common, but demyelination does not occur.

Eleven strains of virus were isolated in adult mice inoculated with brain, CSF, blood, stool, and throat washings obtained from clinical cases (6). A strain was sent to Casals at the Rockefeller Institute, who found it to be related to Mengo virus and encephalo-

myocarditis virus (EMC) (10). It is thus possible that these strains represent mouse viruses and are not involved in the etiology of the disease. Nevertheless, the possibility that this virus is, indeed, the cause of the bizarre CNS illness encountered in the Vilyuisk area cannot be excluded.

SUMMARY

It is widely accepted in the U.S.S.R. that the virus of tick-borne encephalitis, on occasion, persists in the CNS long after the acute illness and causes various progressive lesions which may lead to mental deterioration, paralysis or epilepsia partialis continuo. There are several early reports of TE virus isolation from patients with chronic disease but further confirmation has not been forthcoming.

In a focus along the Vilyui River in northeastern Siberia, a progressive illness of the CNS occurs with a very protracted clinical course. The disease, called Vilyuisk encephalomyelitis, is characterized by mental deterioration and spastic paralysis, and pathologically is an intense pan-encephalitis without demyelination. Several strains of virus were isolated in adult mice inoculated with material from the brain, throat, stool, blood and CSF of patients. This virus is related to Mengo virus and other mouse viruses. It has not yet been established whether the agent came from the human cases or was a contaminant in the mouse colony.

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DISCUSSION

BROTHERSTON: We have been very interested in a syndrome of chronic encephalitis in adult sheep, 3 to 5 years old, in areas where ticks occur. This is unusual as most cases of louping-ill occur in young animals, and the old sheep are usually considered immune. We have isolated virus from the brains from about 30 percent of these cases and they are very similar to louping-ill except that the incubation period in mice is extended, 8 to 11 days, after first inoculation, but reduces on passage. The pathology is similar to that of louping-ill, but there are some differences. We have considered this to be a phenomenon of loss of immunity or failure to become infected as young animals, or, perhaps, infection with louping-ill with a concurrent encephalitis of another sort. The information from the U.S.S.R. on tick-borne encephalitis with chronic sequelae may well be relevant to this problem.

Immunological Characterization of Vilyuisk Encephalitis Virus

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A neurological disease of man has been recognized for over 40 years by clinicians in the Soviet Union in an area along the Vilyuy river in Siberia. The main features of the disease (1) are: an acute phase, often not well-defined, followed by a chronic stage, usually of long duration, in which the main manifestations consist of pronounced rigidity and tremors, cranial nerve damage, especially ocular and vestibular, focal paralyses, muscular atrophy, and psychotic changes. In the 3 to 5 percent of the cases in which death is directly attributable to the disease there is a terminal period of coma. With rare exceptions, the disease affects only persons of the Yakuts ethnic group; it occurs generally between the ages of 16 and 50 years and is more common in females.

Viral strains, presumably identical, have been isolated from clinical cases by inoculation of either blood, cerebrospinal fluid, feces, or brain tissue into white mice by different routes (2); the virus has been reported to be distinct from and unrelated to mouse encephalomyelitis (MEM), lymphocytic choriomeningitis, Japanese encephalitis, and equine encephalitis viruses (3).

The investigations in our laboratory were carried out with strain V-1, supplied by Dr. Sarmanova, which had been isolated from the cerebrospinal fluid of a patient in the chronic stage; the strain when received had had 41 passages in mice.

Effect of sodium deoxycholate.—After incubation for 1 hour at 37° C. with 1 percent sodium deoxycholate, the titer of a suspension of Vilyuisk virus by the intracerebral route in young adult mice was $10^{-6.7}$ LD₅₀/0.03 ml, while the titer of another sample of the same suspension similarly incubated, but without the chemical, was $10^{-6.2}$ LD₅₀/0.03 ml. In being fully resistant to the action of sodium deoxycholate, the

virus met one of the essential criteria for inclusion in the enterovirus group.

Antigenic properties.—The preparation of a complement-fixing antigen had been reported (2) and was easily confirmed. Active antigens with titers in the range between 1 : 64 and 1 : 512 were obtained from brain tissue of newborn mice intracerebrally inoculated with the virus; these antigens consisted of either a 10 percent suspension of tissue in physiological saline or of an acetone-precipitated suspension of the tissue in isotonic sucrose (4). The saline extracts, but not the acetone precipitate, agglutinated sheep erythrocytes at 4° C., and not at 22° or 37° C. The agglutination was coarse and similar to that given by strains of mouse encephalomyocarditis virus (EMC); considering the 10 percent suspension equivalent to undiluted antigen, the titer of the preparations was of the order of 1 : 80 to 1 : 160, when tested with a 0.2 percent suspension of packed cells. Vilyuisk virus antigens have thus far not agglutinated either human or goose erythrocytes. The sensitivity of the hemagglutination inhibition was low; therefore the test has not been very useful for antigenic studies.

Antigenic relationships with other viruses.—Owing to the facts that Vilyuisk virus was not inactivated by sodium deoxycholate, that its isolation was done by inoculation of specimens to mice and that the virus has been maintained in mice, the search for possible antigenic similarities or relationships was immediately directed toward viruses of murine origin. In addition, investigations for possible relationships with arboviruses and a few other viruses available were carried out as a routine procedure. Except as described hereafter, no relationship was found between Vilyuisk virus and other agents.

Determination of serologic relationships with MEM and EMC viruses was done by complement-fixation and neutralization tests. In the complement-fixation test simultaneous dilutions of sera and antigens were used. The sera were derived from mice repeatedly inoculated, 4 or 5 times, with viral suspensions. The result of a test is shown in table I.

TABLE I.—Complement-Fixation Test

Antigen	Serum		
	Vilyuisk	MEM	EMC
Vilyuisk	128	32	0
MEM	16	256	0
EMC	4	8	64

Reciprocal of serum titers; 0 indicates no fixation at dilution 1:4, lowest used.

MEM, mouse encephalomyelitis; EMC, mouse encephalomyocarditis.

The result of this and similar tests showed that Vilyuisk virus is related to both MEM and EMC viruses, being much closer to the former than to the latter.

By neutralization test, using samples of the same mouse sera that cross-reacted by complement fixation, no clearcut cross-reaction was found between any two of the three viruses compared. The homologous titers, in table II, with the exception of EMC, were low; this may explain the inability to demonstrate cross-reactions. Nonetheless, the test permitted easy differentiation of the agents.

TABLE II.—Neutralization Test

Serum	Virus					
	Vilyuisk		MEM		EMC	
	Titer	NI	Titer	NI	Titer	NI
Vilyuisk	4.6	1.7	5.6	1.0	8.7	0
MEM	6.6	—0.3	4.5	2.7	8.4	0.3
EMC	6.6	—0.3	6.5	0.1	4.8	3.9
Normal, control	6.3		6.6		8.7	

Titer expressed as the log of the reciprocal of the dilution giving the 50 percent lethal dose. NI, log neutralization index.

Since it was found that the cross-reactions between Vilyuisk and MEM viruses were consistently more pronounced than those between Vilyuisk and EMC, and since a distant relationship between MEM and EMC has been reported (5), the possibility was con-

sidered that Vilyuisk virus might be a strain of MEM distinct from the GD 7 strain with which the work reported thus far had been done. To investigate this possibility, a comprehensive study was carried out by complement-fixation test of the cross-reactivity between Vilyuisk and several available strains of MEM virus. In addition to strain GD 7, the following were included in the study: GD 1 and FA, which along with GD 7 had been isolated in New York; strain ASK 1, isolated in Tokyo; and strain VIE 415_{ht}, isolated in Vienna. The results of several tests have been combined and are shown in table III.

TABLE III.—Complement-Fixation Tests

Antigen	Virus	Strain	Serum					
			Vil	GD7	FA	GD1	Ask	Vie
Vilyuisk		V-1	128	32	8	8	32	0
MEM		GD7	32	256	128	32	64	32
		FA	32	256	256	64	64	4
		GD1	32	128	64	64	128	8
		Ask	16	128	64	32	128	8
		Vie	16	128	64	32	64	16

See table I for explanations.

Table III shows that, by complement-fixation test, Vilyuisk virus is related to all strains of MEM virus with which it was compared, but is nevertheless easily distinguishable from them. The MEM virus strains show a degree of heterogeneity. The extreme differences between the titers of an MEM serum with its homologous strain antigen and a heterologous one are in the ratio of 1 to 4, being usually 1 to 2; on the other hand, the differences in titers with homologous and heterologous antigens given by Vilyuisk and MEM sera are in the ratio of 1 to 4 at least, being usually 1 to 8 or greater. In other words, Vilyuisk virus, strain V-1, differs from all strains of MEM virus used to a greater extent than any two strains of MEM differ from each other.

DISCUSSION

The circumstances surrounding the reported isolations of strains of Vilyuisk virus, in addition to its resistance to the action of sodium deoxycholate and to its immunological relationship with strains of MEM virus, might lead to the consideration that the virus did not derive from the human materials inoculated but was instead a contaminant derived from the recipient mice. Whether or not this is the case cannot be established with the materials available in this laboratory. An

epidemiological investigation has been reported by Sarmanova and Chumachenko (6), who detected neutralizing antibodies against Vilyuisk virus in convalescents, as follows: of 12 persons bled within the first year after onset, 2 were positive; of 26 bled between 2 and 5 years after onset, 9 were positive; and of 16 persons bled from 6 to 15 years after the acute illness, 6 were positive. In contrast, of 34 persons not affected and not residing in the zone who were tested, only one had antibodies; this individual had been in close contact with some of the patients. By complement-fixation test, of a total of 75 patients either in the chronic stage or bled between 2 and 6 months from the acute phase or with residuals, 36 were positive with titers from 1 : 4 to 1 : 32; of 18 controls, i.e., persons not affected, only 1 was positive.

These results of Sarmanova and Chumachenko lend support to the view that the agent plays an etiological role in human disease. More such studies are needed, including an investigation of the presence of antibodies against the virus in patients' convalescent sera and comparison of these sera with the acute-phase specimens or with sera of persons who have not been affected.

It should be borne in mind that the relationship of Vilyuisk virus with murine agents is not a reason for rejecting the proposed etiological role of the agent; lymphatic choriomeningitis and the Mengo strain of mouse encephalomyocarditis are examples of human pathogenic viruses that are also associated with mice.

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DISCUSSION

KIBRICK: I am not quite clear as to whether it is fairly definite that the isolates obtained from brain, blood, spinal cord, and stool represent the same agent, or whether it is one isolate from one particular site that you have worked with?

CASALS: According to reports that I have had from Dr. Sarmanova, they have isolated quite a number of strains, 8 or 10 at least, and as far as I know they have settled the question that they are identical. The question about whether or not their mice are infected still remains. All I can say is that, if it is a mouse pathogen, it is distinct from other enteroviruses of mice that are found in such distant places as Central Europe, Japan, and New York.

KIBRICK: It is possible for a person who has had an enterovirus infection in the past to acquire the same virus again, if it were prevalent in the area. The agent would now induce either a transient alimentary tract infection or just pass through. In either instance the agent would be demonstrable in the stool. It would bear no relationship, however, to any chronic illness which might also be present in this patient.

CASALS: Yes, if it were to be found only in the gut or the blood, but the spinal fluid—that is a strange place to have a virus, particularly when many of the enteroviruses are not often found in the spinal cord.

GAJDUSEK: In view of that, I would like to ask Dr. Thormar to tell us for how long he recovers visna from spinal fluid.

THORMAR: For as long as a year, and in the presence of antibody.

BRODY: This virus was isolated on several occasions by directly feeding the mice the pathological material, blood, or stool, or spinal cord, so there is no doubt that it was an enterovirus in that sense.

CASALS: Repeated isolations have been reported in Vilyuisk disease from blood and spinal fluid for many months after what has been considered the onset of disease. I do not dismiss the agent isolated as the etiologic agent; it is just that it has not been proven yet.

HOTCHIN: I thought this reminded us all about the scepticism that existed for a long time about human cases of LCM, again a mouse virus. But when you have one or two human clinical cases and then you do isolate the virus and get good titers, as happened in my laboratory with LCM virus, you become extremely convinced that the agent can cause human

disease. When we did an antibody survey we found 5 human cases out of the laboratory population of approximately 50 that showed the fluorescent test to be positive. Everyone of them was in the group working with LCM. So, although most mouse viruses may be either nonpathogenic for man or lead to only very slight inapparent infection, perhaps some others can be the cause of a definite disease with sequelae.

CASALS: There is also the example of Mengo which is a pathogen of mice and produces disease in man.

GAJDUSEK: I would like to ask Dr. Hotchin if he would comment in regard to his mice that have persistent tolerant infection with LCM. As they age and get very old, have you looked serially for various types of antibodies to LCM by a variety of techniques? I was wondering whether they might, at that stage, begin to develop complement-fixing or

neutralizing antibodies, having borne the virus all their lives.

HOTCHIN: We have never been able to find any LCM antibody in mice that are carrying LCM virus.

MACKENZIE: In the epidemic of hemorrhagic fever in Bolivia we have found that laboratory hamsters inoculated with the virus which causes the disease do develop CF antibodies and neutralizing antibodies and then, about 2 weeks after inoculation, they begin excreting the virus in their urine and have continued to do this so far for about 9 months; we have found also that *Colomys callosus*, the rodent that we feel is the reservoir in San Joaquín, is doing the same thing, now up to about 2 or 3 months.

BRODY: As far as the Vilyuisk encephalitis is concerned, it is a very odd disease too, to involve so much and yet go on for so long. It is obvious that whatever is going on is going on slowly and makes it appropriate for this symposium.

The Incubation Period of Viral Encephalitis¹

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The clinical manifestations and severity of viral infections are largely predetermined in the incubation period, for it is during this interval of virus proliferation and activation of host defense mechanisms that the outcome of infection is decided. During the incubation period of viral encephalitis the virus has been assumed to multiply in extraneural cells, to gain access to the central nervous system (CNS) along hematogenous or neural pathways, to invade nervous tissue, and to penetrate susceptible cells within the nervous system. The subsequent destruction or functional derangement of these cells gives rise to the clinical disease of encephalitis, but the interruption of this chain of events at any level by cellular or humoral factors may prevent disease.

In the case of slow virus infections of the CNS the incubation period is of even greater interest. These prolonged incubation periods cannot be viewed in simple terms of a race of virus against antibody response or of a time interval required for virus to grow from site of inoculation to end organ. Indeed, it is difficult to conceive of the cellular events and interaction of virus and host that give rise to an incubation period lasting months or even years. Studies in cell culture systems may give some clues to possible mechanisms. The equilibrium of cell generation time and inefficient virus infection shown by Stoker (1) in the Herpes simplex-HeLa cell system might afford a model for latent herpes infections. The development of a "cold sore" might result when this equilibrium of virus and epithelial cells is disturbed by fever, trauma, or

even emotional stress. Such speculation, however, could not explain the latent herpes infection of brain described in rabbits (2), unless virus were infecting cells which were actively dividing. Conversely, the endosymbiotic relationship of virus and cell shown by Fernandes, Wiktor, and Koprowski (3) in a fixed rabies-rabbit endothelial cell system provides a model for a possible mechanism of latent infection even in neural tissue. However, it is difficult to transpose this endosymbiotic relationship to the long incubation period of rabies or other slow virus infections. To produce disease the symbiosis would need to be disrupted by some environmental or host factor, and, therefore, the incubation period would be of variable duration instead of the rather consistent, long incubation periods seen with these infections.

These and similar in vitro studies may provide interesting leads, but the definitive answers must be provided by experiments in animals. The complexity of the intact animal with its varied tissues and specialized cell populations frustrated early investigations into the pathogenesis of even acute virus infections. Early studies of pathogenesis utilized primarily two techniques. The first was the histologic method such as employed by Goodpasture (4) in the study of herpes virus encephalitis. This method was based on the assumption that inclusion bodies, inflammation, or cell necrosis mirrored the sites of virus replication. This assumption that sites of virus growth can be equated to sites of histologic alteration has not been found to be valid (5-8). The second was the titration method used by Fenner (9) in the study of the pathogenesis of ectromelia. Tissues at various stages of the incubation period were removed and titrated for virus. This method may indicate which organs are supporting

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virus growth but not which cell types are infected. Furthermore, the presence of a viremia during the experimental infection may totally obscure results, since virus recovered from organs may represent blood-borne virus in transit through the organ rather than virus multiplication within the organ. It is no wonder that the introduction of tissue culture techniques led virologists to retreat to the study of virus infections of homogeneous cultured cells.

The introduction of fluorescent antibody staining by Coons (10) has now provided a method with which cellular events of the incubation period can be reconstructed with some degree of precision. The author has been utilizing this method in studies of the pathogenesis of acute viral encephalitis; the results of these studies will be reviewed in order to demonstrate the potential of the method for *in vivo* investigations. The possible application of fluorescent antibody staining to the study of slow and latent virus infections will then be discussed.

Incubation Period of Acute Viral Encephalitis Studied by Fluorescent Antibody Staining

Sindbis Virus Encephalitis.—The first type of pathogenesis to discuss is that of an acute hematogenous spread of virus to the brain such as occurs with arthropod-borne virus infections. Virus, injected by a vector, presumably establishes growth in some ex-

traneural tissue; a viremia develops; and by some mechanism virus crosses the elusive blood-brain-barrier. Virus must then penetrate susceptible cells within the CNS and involve sufficient cells to produce symptoms. Sindbis virus, a group A arbovirus, produces encephalitis in suckling mice following extra-neural inoculation with an incubation period of only 48 to 72 hours. In contrast to the subject of this workshop, Sindbis presents a problem in explaining the completion of the above steps in a very short incubation period.

Fluorescent antibody staining showed that the initial virus multiplication in suckling mice took place within muscle cells and fibroblasts at the site of subcutaneous inoculation. A viremia of greater magnitude than could be accounted for by virus within the inoculum developed within 6 hours; this was a plasma viremia which persisted at high titer until death. There was no evidence that growth within or transport by blood cells contributed to the viremia. Within 24 hours antigen was present in striated muscle cells throughout the body (fig. 1). Fluorescent smooth and cardiac muscle cells were also found as were fluorescent endothelial cells of small vessels. Other extra-neural cells and tissues were free of specific fluorescence. Between 18 and 24 hours after inoculation fluorescence was found in the endothelium of small cerebral vessels (fig. 2). This fluorescence preceded the development of antigen in neural and glial cells.

Photomicrographs of mouse tissues taken during the incubation period of viral encephalitis and stained with fluorescent antibody:

FIGURE 1. Sindbis virus. Cross section of paravertebral muscle of a 2-day-old suckling mouse 24 hours after subcutaneous inoculation of Sindbis virus. Fluorescence is predominantly in peripheral cytoplasm corresponding to sarcoplasm. $\times 340$

FIGURE 2. Sindbis virus. Section of brain of 2-day-old suckling mouse 20 hours after subcutaneous inoculation of Sindbis virus. Fluorescence is limited to an endothelial cell of a small cerebral vessel and neural and glial cells of brain are still free of fluorescence. Vessels are filled with India ink for identification. $\times 340$

FIGURE 3. Herpes simplex virus. Sagittal section of head of a 1-week-old suckling mouse 4 days after intranasal inoculation of herpes simplex virus. Junction of trigeminal nerve (left) with gasserian ganglion (right) is shown. Fluorescent cells in nerve are predominantly Schwann cells, and there is early perinuclear fluorescence of several ganglion cells. $\times 85$

FIGURE 4. Herpes simplex virus. Section of lung of a 1-week-old suckling mouse 4 days after intranasal inoculation of herpes simplex virus. A large focus of fluorescent cells is shown. $\times 85$

FIGURE 5. Rabies virus. Section of cerebellum of an adult mouse 5 days after intracerebral inoculation with the CVS strain of fixed rabies virus. Fluorescence is limited to the cytoplasm of Purkinje cells with granules of fluorescence in the dendrites (left) and perinuclear cytoplasm (center), but with no fluorescence in axons, which extend through the granular layer (right). $\times 340$

FIGURE 6. Rabies virus. Section of a left lumbar dorsal root ganglion of a 1-week-old suckling mouse 4 days after subcutaneous inoculation of fixed rabies virus in the left footpad. Early cytoplasmic foci of fluorescence are present in numerous ganglion cells. $\times 340$

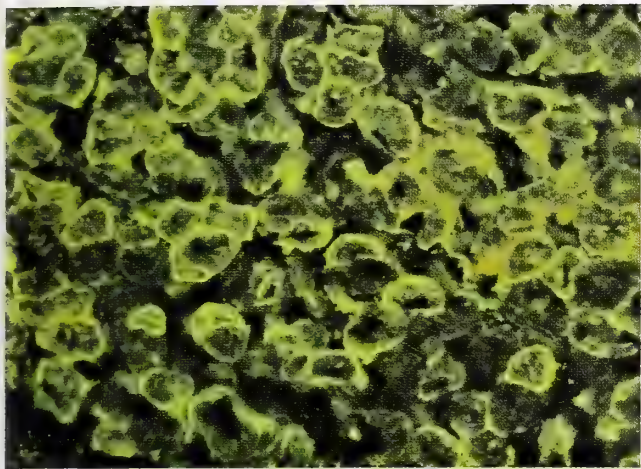


FIGURE 1

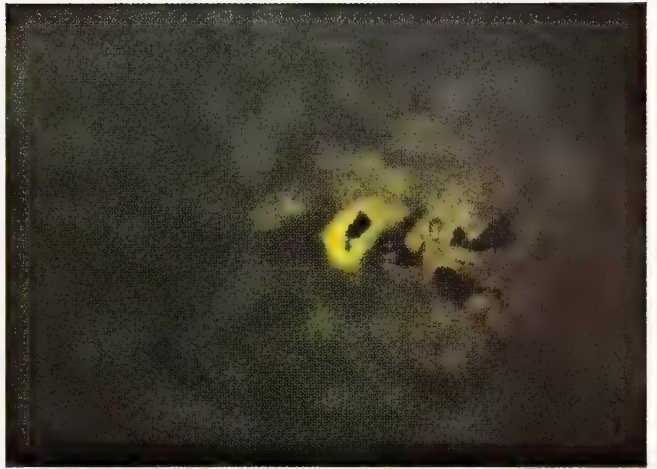


FIGURE 2

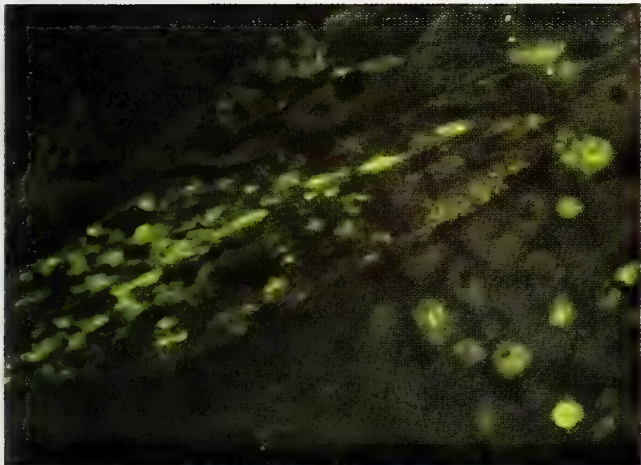


FIGURE 3

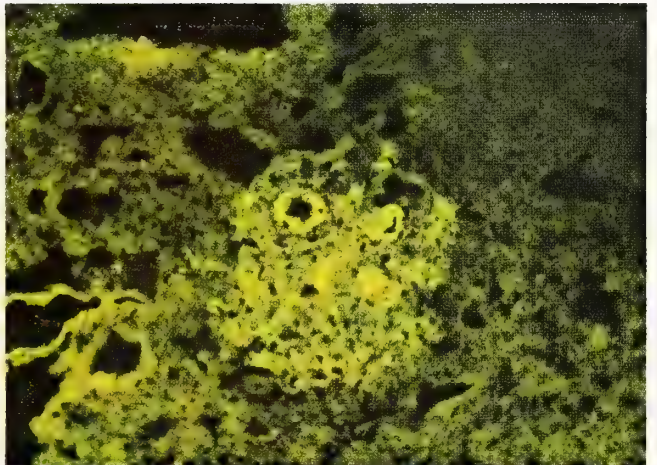


FIGURE 4

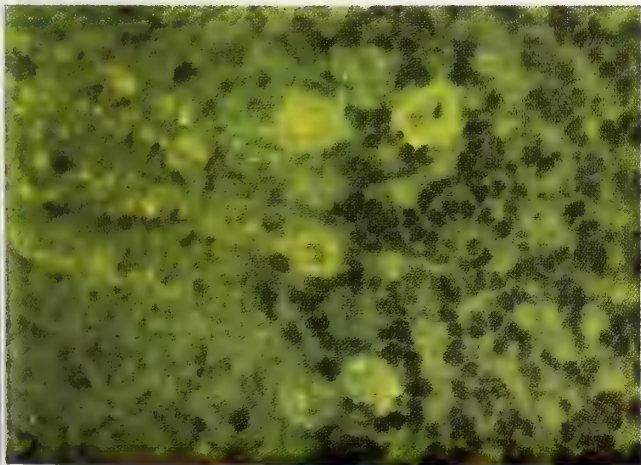


FIGURE 5

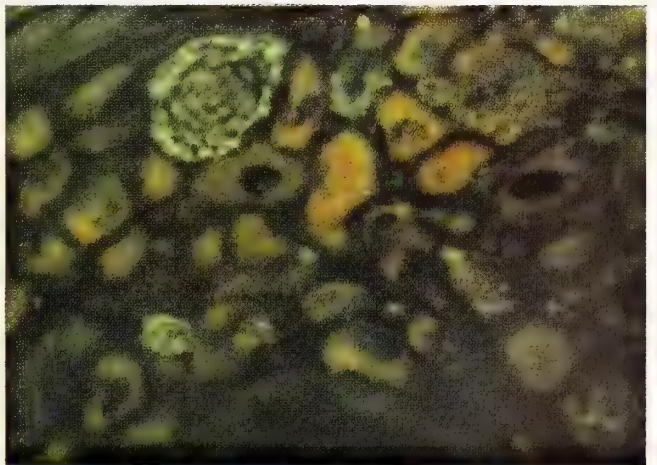


FIGURE 6

Forty-eight hours after inoculation there was widespread fluorescence of neurons and glial cells of the brain, and signs of clinical encephalitis developed.

Thus, muscle proved to be the major site of extraneural growth, virus replicated in muscle or vascular endothelium presumably seeded into the blood, and virus spread to the susceptible CNS cells by growth through the small cerebral vessels (6).

Herpes Simplex Virus Encephalitis.—Most viruses show somewhat longer incubation periods even in small laboratory animals, and the pathway to the CNS is not always hematogenous. Under certain circumstances herpes simplex virus spreads to the brain of suckling mice, via peripheral nerves (7). For example, after subcutaneous inoculation of suckling mice, myelitis developed after an incubation period of about 5 days. During this incubation period no virus was detected in viscera or blood by fluorescent antibody or titration methods. With fluorescent antibody staining development of antigen was detected only in a few scattered elongated cells in subcutaneous tissue at the inoculation site. Small subcutaneous nerve fibers also contained fluorescent cells within 24 hours after inoculation. This infection of endoneural cells could be followed over subsequent days ascending via the sciatic nerve to the corresponding dorsal root ganglia and spinal cord segments. The spinal cord was initially infected primarily in the ipsilateral posterior column corresponding to the entry zone of the afferent fibers; but, as infection spread within the spinal cord, neurons and glial cells in all quadrants were infected, and infection spread both caudally and rostrally. A similar infection along the first and fifth cranial nerves was observed after intranasal inoculation of herpes simplex virus in suckling mice (fig. 3). However, after intranasal inoculation there was also growth of virus in lung (fig. 4) with viremia and hematogenous spread of virus.

Thus, the incubation period of herpes simplex encephalitis, in some instances, represented the time of virus growth up endoneural cells of nerves and subsequent cell to cell spread of virus within the nervous system until sufficient cell damage had taken place to produce signs of myelitis or encephalitis (7, 8).

Fixed Rabies Virus Encephalitis.—The laboratory attenuated fixed strains of rabies are not slow viruses as are their antecedents, the street strains of rabies. After intracerebral inoculation of the CVS strain in mice, disease developed after an incubation period of 5 to 6 days, and fluorescent antibody and electron microscopic studies suggested that this interval of time was needed for the slow development of virus within

the cytoplasm of infected neurons (11). Two to 3 days after inoculation small flecks of fluorescence were seen in the perikaryon and dendrites of susceptible neurons, and over the subsequent 3 to 4 days these fluorescent areas enlarged until the perikaryon and dendrites were confluent fluorescent at the end of the incubation period (fig. 5).

Although fixed rabies virus was not demonstrated in any cells in vivo except neurons, the agent gained access to the CNS of mice if sufficiently large amounts of virus were inoculated extraneurally. Encephalitis after extraneural inoculation developed after an incubation period of 6 to 7 days. Studies involving amputating of limbs and cutting of nerves confirmed previous reports that virus ascended along peripheral nerve pathways. However, no fluorescence of endoneural cells developed at any time and, unlike the time required for herpes simplex virus to reach the spinal cord, fixed rabies penetrated to the spinal cord within 24 hours. This suggested that it may reach the cord without extraneural replication, but the mechanism by which fixed rabies virus spreads centripetally along nerves remains obscure. Three days after inoculation fluorescent flecks were found in neurons of the lumbar ganglia and cord (fig. 6), and by 4 days susceptible neurons throughout the neuraxis showed evidence of infection. Thus, the incubation period of fixed rabies virus encephalitis after extraneural inoculation was only about 24 hours longer than that after intracerebral inoculation. Even after extraneural inoculation the incubation period of fixed rabies virus encephalitis represented, in large part, the time required for virus to develop within the susceptible neurons (12).

Possible Application of Fluorescent Antibody Staining to the Study of Slow or Latent Virus Infections

Before concluding that immunofluorescent techniques may be the panacea for the study of slow or latent virus infections, let us examine some shortcomings as well as advantages of the method. The introduction of isothiocyanate, improvements in methods of reducing nonspecific fluorescence, the availability of commercial conjugates, etc., have simplified the technical aspects of fluorescent antibody staining, but it still can be a capricious technique when applied to histologic sections.

The staining of virus-infected cell cultures with fluorescent antibody presents a relatively simple task since one is dealing with a monolayer of homogeneous cells. Sections of tissues, however, present a mosaic

of different cells cut at different angles and exposing a variety of antigens. This, of course, presents problems in nonspecific fluorescence. Also the cutting of frozen unfixed tissue is often plagued by ripples and tears of the section from which stain does not satisfactorily wash. In addition, the study of encephalitis involves the staining of neural tissue which presents a special problem in the nonspecific fluorescence of neurons. These problems encountered in the studies of acute viral encephalitis can be overcome by care in the preparation of sections, by removal of sufficient nonspecific staining from the conjugate, and by obtaining of a conjugate system of sufficient brilliance so that individual infected cells can be seen against the varied background.

Antisera and Conjugates.—In the author's experience the most crucial factor in fluorescent antibody staining has been the obtaining of a satisfactory antiserum; my method for this has been pragmatic rather than scientific-trial and error. Initially herpes simplex virus antisera were prepared in rabbits and chickens. These sera were then screened using neutralization tests in eggs on the assumption that sera with the higher antibody titer would be better for fluorescent staining. This did not prove to be the case since rabbit and fowl sera with high neutralizing antibody titers did not provide adequate fluorescence. Human sera were then tested and again neutralizing antibody failed to correlate with fluorescence. The serum used in the above studies was that from a colleague with recurrent herpes labialis whose serum showed a neutralizing titer of only 1:40. Similarly, fowl sera with high antibody titers against several arboviruses proved useless in staining for these agents, and the studies of Sindbis virus utilized immune mouse ascitic fluid. This failure of neutralizing antibody to correlate with "fluorescent antibody" may have some relevance to the investigation of slow viruses. For example, the failure to demonstrate neutralizing antibody against scrapie virus does not eliminate the possibility that conjugates prepared with sera of immunized animals might yield fluorescence of infected cells.

Histologic Methods.—In each of the studies mentioned above tissues were snap-frozen in containers in a bath of liquid nitrogen or CO₂ and alcohol, stored at -20° C. for variable times up to a week, cut on a simple rocker microtome in a -20° C. box, and fixed with acetone for 10 minutes at room temperature. In each of the above systems fixation with acetone, formalin, and methanol (at -60° C.) as well as unfixed material were compared. In each of these cases acetone fixation provided the best re-

sults, but this has not been a universal experience (13). Whenever possible the direct staining method (i.e., conjugation of the antiserum with fluorescein isothiocyanate) was employed. Being a simpler system than indirect staining it involves less time and less source of error. However, indirect staining is a more sensitive method and may increase the degree of fluorescence. Coons was estimated 10 fold enhancement of fluorescence with the indirect system (14). It was for this reason that it was necessary to use indirect staining (i.e., human antiherpes serum and fluorescein-labeled goat anti-human-globulin serum) in studies of herpes simplex (7, 8).

Day to day variations in the quality of staining is difficult to avoid, and this necessitates the cutting and staining of matching sets of uninfected tissues with each day's work.

Nonspecific Staining.—Nonspecific staining in fluorescent antibody studies refers not only to the staining caused by unreacted fluorescent material but also the staining due to various conjugated serum proteins and unwanted conjugated antibodies. Thus, any staining other than the specifically desired reaction of labeled antibody with corresponding antigen is referred to as nonspecific. Also some tissue components such as the keratin layer of skin, cartilage, eosinophils, gastric mucosa, salivary gland, and neurons tend to fluoresce. Unreacted fluorescein and unwanted labeled proteins can be removed with a variety of absorbing materials, but I have not found such methods as Sephadex or electrophoresis to be more satisfactory than the simple method of absorbing with acetone-dried organ powders.

Unfortunately the treatment of conjugates to remove nonspecific fluorescence also results in some diminution of desired fluorescence. Compromise must be accepted.

Sensitivity of Technique.—A major problem of applying fluorescent antibody staining in the study of slow, latent, and temperate virus infections in animals is the uncertain sensitivity in the technique. Although sensitivity of the method has been calculated for pneumococcal antigen (14), no quantitative studies have been carried out with viruses. The failure to demonstrate fluorescence in cells, such as the absence of fluorescence in endoneural cells in the studies with fixed rabies virus, does not establish that virus was not present or multiplying within the cells. Virus may be present in amounts below the threshold of the system. In the studies above fluorescence was not detected until titers of virus within tissues exceeded 10³ plaque-forming or pock-forming units per gram

of tissue. Therefore, the search for fluorescent cells in the brains of rabbits with latent herpes simplex virus infections might be not only time consuming but unrewarding. The same may well be true of a search for cells containing street rabies virus during its long incubation period. However, sensitivity is based on the concentration rather than the overall quantity of antigen, and if a small quantity of virus is concentrated in one focus within one cell it may be found.

In conclusion, the application of fluorescent antibody staining to the study of pathogenesis of slow and latent virus infections may present many problems. The obtaining of a satisfactory antiserum will be a crucial factor and may of necessity depend on trial and error selection. However, the absence of demonstrable antibody or of a characterized antigen, for that matter, should not be a deterrent to employing the methods; it is probably the only technique with which an unknown antibody can be used to search for an unknown antigen. For example, otherwise undemonstrable antibody was titered using an uncharacterized antigen in the early studies with primary atypical pneumonia (15, 16) and previously unknown antigen was demonstrated in rheumatic hearts using previously unknown antibody in the serum of rheumatic fever patients (17). No other method currently available has the potential to add so materially to our understanding of the pathogenesis of virus disease. Application of immunofluorescent methods to the studies of slow and latent virus infections may provide quicker and more precise answers to the unknown mechanisms by which these agents spread and sequester themselves within the animal host.

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DISCUSSION

Q: Do you have any idea what part of the virus is fluorescing in your preparations? Does it have to be whole virus, or can you accept viral particles? This is pretty critical in slow virus work.

JOHNSON: When you immunize with whole virus, you do not know which of the components you are really forming antibody to. I think this can be best exemplified in the case of herpes simplex where

it is known that the nucleic acids form in the nucleus and then pick up the protein coat as they cross the nuclear membrane into the cytoplasm. Now, in the original study on fluorescent antibody in Herpes simplex done by Lebrun in 1956, she described some fluorescence in the nucleus, followed by a brighter fluorescence in the cytoplasm (*Virology*, 2, 496, 1956). The next two or three people who did fluorescent antibody work in cell culture systems with Herpes simplex described only cytoplasmic fluorescence. Lebrun apparently had an antibody against both the internal and the external structures of Herpes simplex virus. She got fluorescence in both places; the other workers and I apparently had antibody only against the coat proteins. It is only with this kind of indirect evidence that you have any idea of what you have antibody against.

CASALS: The question of the relative titers with the different serological methods is a question that comes up all the time. Now, the system that you have been using, Sindbis, you can study with complement fixation, fluorescent antibodies, hemagglutination-inhibition, and neutralization tests, and they will often vary, as in the work that we have done in our laboratories. Your complement-fixa-

tion titer and FA titer are very similar, and if you have a mouse serum that will titrate, say, 1:128, the same serum, if you test it by hemagglutination-inhibition, may give you a 40-fold increase of titer, 1 to 5,000 or 1 to 10,000.

HOTCHIN: It may be that a different antibody is involved; that is, the one that is successful for fluorescent antibody work is different from a neutralizing or ordinary complement-fixing antibody that you titrate. In reference to the ability to make a diagnostic case with fluorescent antibody, I was very interested when you mentioned the human anti-herpes serum, and I wonder if you have ever used any human sera to follow the tissue growth of virus.

JOHNSON: When I first tried human serum I used sera from patients after primary herpetic infection. However, it was only sera from patients with recurrent herpetic lesions, particularly people who had them quite often, that have been satisfactory, and yet these people notoriously have rather low titers of neutralizing antibody and rather low CF titers. I think with this immunofluorescence method we are measuring some other antibody or quality of antibody.

Endosymbiotic Relationship Between Animal Viruses and Host Cells. A Study of Rabies Virus in Tissue Culture¹

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Persistent infection of various tissue culture systems with rabies virus has been described in several communications (1-3). In the course of these investigations, it became apparent that, depending on the tissue culture system used and on the stage of cultivation, rabies antigen may be present in all cells of the culture but growth of the infected culture does not seem to be impaired. This situation seems to be particularly characteristic for a rabies-infected rabbit endothelium cell line (1). This paper presents an analysis of the virus-host cell relationship in such a system.

MATERIALS AND METHODS

Virus. The Challenge Virus Standard (CVS),² a fixed rabies virus propagated in mouse brain (MB-CVS), was originally used to infect the rabbit endothelium (RE) cell system (1), and the Pitman-Moore (PM) strain of fixed rabies virus propagated in rabbit brain (RB-PM)² was used in the interference experiments. The two virus pools were maintained at -70° C. as a 20 percent suspension of the infected brain tissue in 50 percent normal calf serum in distilled water. A strain of vesicular stomatitis virus (VSV) propagated in MK-2 cells in tissue culture was also used in the interference experiments.

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² Supplied by the National Institutes of Health, Bethesda, Md.

Tissue Cultures. Monolayer tissue cultures of rabbit endothelium cells (4) and rabies virus-infected rabbit endothelium cells (RE-CVS) (1) were propagated in milk dilution bottles and subcultivated twice weekly. The nutrient medium consisted of modified Eagle's basal medium (5) in Earle's balanced salt solution supplemented with 10 percent inactivated calf serum and containing 25 ml. of 5.6 percent sodium bicarbonate, 10⁵ units of penicillin and 10⁵ µg of streptomycin or 5 × 10⁴ mg. of aureomycin per liter.

For cytological observations and immunofluorescent antibody staining (FA) described below, the cells were grown in the same medium on coverslips in Petri dishes and incubated at 37° C. in a humidified CO₂ incubator.

A stable line of neonatal hamster kidney fibroblasts, BHK-21, clone 13, referred to as C-13 (6), propagated in the same nutrient medium was used for some of the in vitro titrations and for the infectious center experiments (see below).

(a) **Titration of virus by FA in tissue culture.**—Ten-fold dilutions of the virus were prepared for titration in nutrient medium and 1 ml. of each dilution was mixed with 5 ml. of a C-13 cell suspension containing 5 × 10⁵ cells, which were plated out in Petri dishes containing coverslips and incubated at 37° C. for 6 days and stained. The highest dilution of virus causing staining of cells by FA was considered the titration endpoint.

(b) **Infectious centers.**—C-13 cells were grown in Petri dishes (50 mm.) in monolayers over coverslips and seeded with a suspension of RE-CVS cells containing approximately 1,000 cells per dish. Taking

into consideration the dimensions (11×22 mm.) of the coverslips, approximately 100 RE-CVS cells were seeded for each coverslip. After 18 hours of incubation, one coverslip was removed from each Petri dish and stained by FA, and the number of dispersed fluorescing RE-CVS cells was counted. After 6 days' incubation the other coverslips were removed and stained by FA, and the number of fluorescing cell aggregates containing more than 10 cells counted.

(c) **Plating efficiency.**—Five hundred RE and RE-CVS cells were plated out into 50 mm. plastic Petri dishes for each series of tests for plating efficiency. The plates were cultivated for 16 days, the viable colonies in each plate were counted, and the number averaged to determine the final result for each particular series.

(d) **Cytological preparations.**—Actively proliferating cultures were selected for chromosome analysis and treated according to the method described by Saksela and Moorhead (7).

(e) **Autoradiography.**—Cultures of RE and RE-CVS cells, grown on coverslips in Petri dishes, were exposed to $2 \mu\text{C}/\text{ml.}$ of H_3 -thymidine (specific activity $3\text{c}/\text{m mole}$). At different times after exposure, ranging from 30 minutes to 7 hours, the coverslips were washed several times in phosphate buffered saline (PBS) and fixed in Carnoy's solution followed by Feulgen's reaction. They were coated with NTB 3 emulsion (Kodak, Rochester, N.Y.), exposed in the dark at 4°C. for 10 days, the film developed in D-19 solution and fixed with acid. To ensure good cellular detail, the cultures were then stained with 5 percent toluidine blue at pH 3.5.

In another series of experiments, the cultures of control and infected cells were exposed to $6 \mu\text{C}/\text{ml.}$ of H_3 -uridine (specific activity $4\text{c}/\text{m mole}$) for periods varying from 30 minutes to 28 hours. The coverslips were washed in PBS, fixed in Carnoy's solution for 30 minutes, and treated with 2 percent percholoric acid for 40 minutes at 4°C. to remove soluble nucleotides. Half of the coverslips in each group were treated with RNase (Worthington) $60 \mu\text{g}/\text{ml.}$ in veronal acetate buffer, pH 7.8, for 2 hours at 37°C. Finally, the coverslips were covered with NTB 3 emulsion, exposed in the dark for 20 days, and treated as in the previous experiment.

Antirabies Sera. Three types of sera were used:

(a) Serum obtained from a man who had, in the past, undergone an antirabies Pasteur treatment (Fermi type of vaccine) and had, in addition, received three booster inoculations of Flury HEP virus. This

serum, which had a neutralization titer of $1/380$ in mice, is referred to here as Human W.

(b) Serum from a man who had received three injections of rabies duck embryo vaccine followed 1 year later by one booster inoculation of the same vaccine. This serum had a neutralization titer of $1/830$ and is referred to here as Human F.

(c) Antirabies gamma globulin prepared in horses at the Metchnikoff Research Institute, Moscow³ with a neutralization titer of $1/1000$ in mice (referred to here as Horse P).

Complement. Blood obtained from young adult hamsters by heart puncture was stored overnight at 4°C. The serum was then centrifuged off, immediately frozen, and used in a 1:10 dilution.

Microscopic observations

(a) **Phase Contrast.**—Coverslip preparations were mounted in closed chambers and observed under phase contrast optics.

(b) **Immunofluorescent Antibody Staining (FA).**—Cells on coverslips were stained directly with antirabies globulin conjugate⁴, using the technique of Coons and Kaplan (8), as modified by Goldwasser et al. (9).

(c) **May-Grünwald Giemsa Staining.**—Coverslips were washed twice in PBS, fixed in Bouin's fixative for 10 minutes, stained with May-Grünwald for 15 minutes and then for 30 minutes with a $1/30$ Giemsa solution in buffered distilled water (pH 6.8). They were then processed following routine procedure for preparation of permanent mounts.

Inoculation of experimental animals

(a) **Mice.**—For titration purposes, 3- to 5-week-old Swiss white mice were injected intracerebrally with serial dilutions of virus. The endpoints were calculated according to the method of Reed and Muench (10). For immunization purposes, 5-week-old mice were injected intraperitoneally with 0.25 ml. of an undiluted suspension of RE-CVS. Three weeks later these mice were challenged either intramuscularly with 0.1 ml. of a 1:30 dilution or intracerebrally with 0.03 ml. of serial dilutions of MB-CVS pools. Non-vaccinated mice of the same age were used as controls.

(b) **Rabbits.**—Five-day-old and young adult rabbits were inoculated intracerebrally with 0.05 ml. of a cell-free extract from RE-CVS cells.

³ Obtained through the kindness of Dr. Martin Kaplan of the World Health Organization.

⁴ Antirabies gamma globulin conjugate supplied by the National Rabies Laboratory, Atlanta, Ga.

RESULTS

Immunofluorescence of Cells Containing Rabies Antigen.—RE cells exposed in suspension to MB-CVS at an input multiplicity of 5 can be grown as monolayers in tissue cultures and maintained for several consecutive transfers without evident degenerative changes (1). Through 93 transfers of the infected cells, there has been no evidence of cytopathic effect (CPE). The presence of viral antigen in the cultured cells was determined by staining RE cells grown on the coverslips with fluorescein-labeled antiserum.

Three days after exposure to the virus pool (MB-CVS), small bright fluorescent granules were observed dispersed throughout the cytoplasm and in a few RE cells the granules were concentrated in the perinuclear zone. On the 7th day, the granules increased in size and formed small, round, or oval-shaped, inclusion-like masses, but the percentage of cells showing fluorescence remained low (1 percent) through the 10th day of cultivation. During subsequent transfers, the number of RE cells showing the presence of intracytoplasmic antigen increased rapidly from the level of 25 percent at the third subculture to 100 percent at the fourth subculture. The persistence of viral antigen in all the cells of the RE-CVS cultures was observed throughout the 15 months' cultivation period,

corresponding to 93 cell transfers (fig. 1). The cytoplasmic inclusions were the same size and shape as those in the cells primarily infected with the MB-CVS pool and as shown in figures 2–5, they were observed in a number of cells in various stages of mitosis.

After May-Grünwald Giemsa staining, the round or oval-shaped inclusions were red and surrounded by a clear halo (fig. 6). Because of their cytoplasmic localization and the percentage of cells showing their presence, these inclusions seemed to be the same as those demonstrated by FA.

Under a phase contrast microscope, no specific lesions were observed in any of the cellular organelles of cells of the RE-CVS series and the inclusions appeared as small, round or oval, uniformly dense masses in the cytoplasm (see fig. 7). Normal filamentous mitochondria were noted around the inclusions. The small fluorescent granules which were detected after staining with FA could not be seen after May-Grünwald Giemsa staining, or observed under the phase contrast microscope.

After several passages, as the CVS became better adapted to growth in the RE cell system, it was possible to titrate the virus in the RE cells which were subsequently stained by FA. Figure 8 shows the results of comparative titrations in RE and C-13

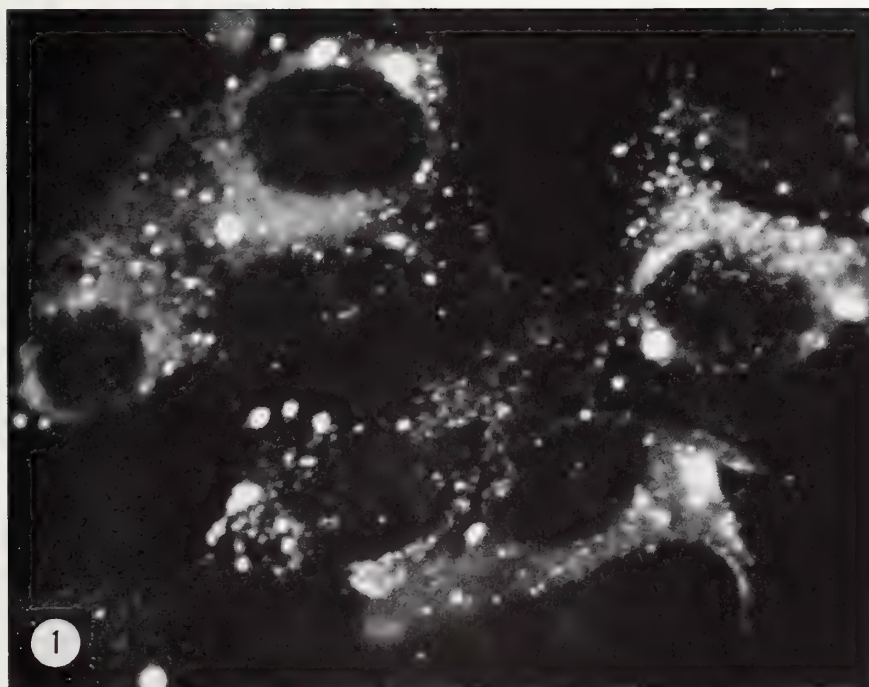
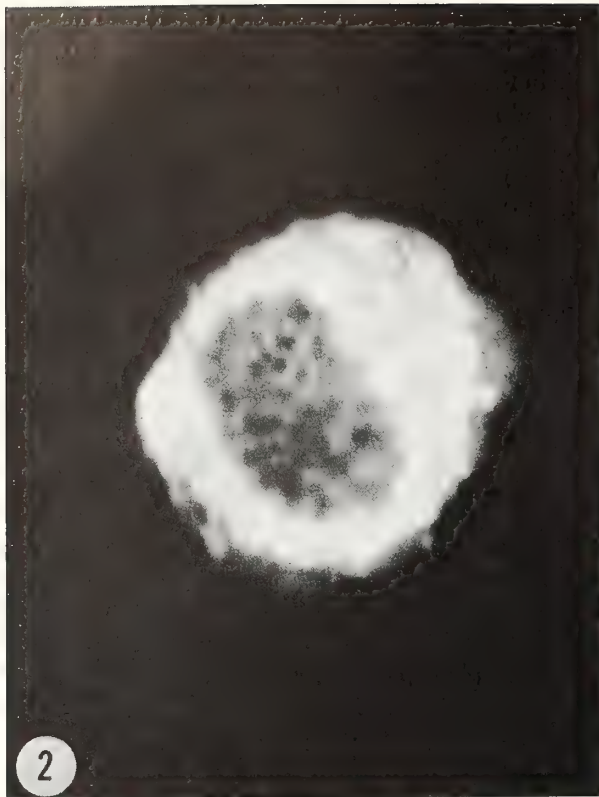


FIGURE 1.—62nd subculture of RE-CVS cells. Note the presence of bright fluorescent granules and small fluorescing inclusions in all cells. Immunofluorescent antibody staining. $\times 1200$.



FIGURES 2 to 5.—53rd subculture of RE-CVS cells. Bright fluorescent granules in various stages of mitosis. Immunofluorescent antibody staining.

FIGURE 2.—Prophase $\times 1600$.

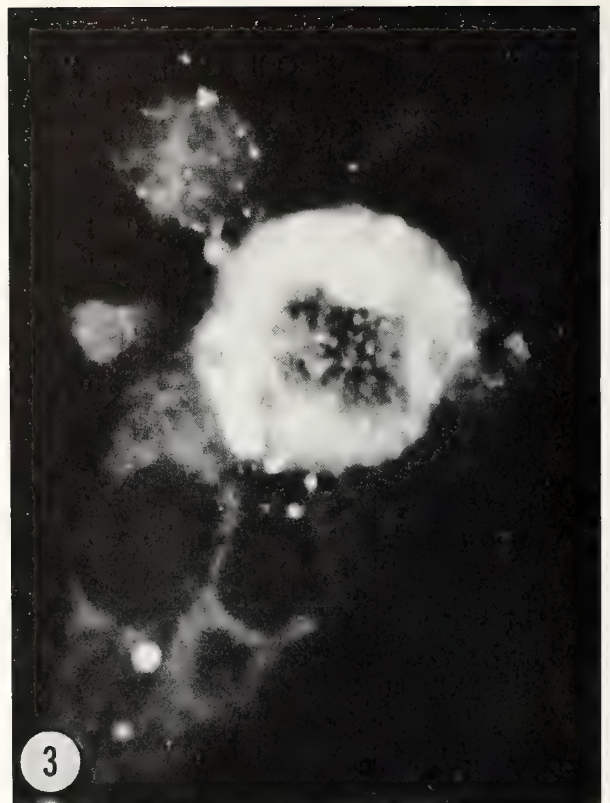


FIGURE 3.—Metaphase $\times 1360$.

cells. As can be seen, the C-13 cells appeared to be equally susceptible to infection with MB-CVS and RE-CVS, and the titer of the RE-CVS in the RE cells was higher than that of the MB-CVS. These data show that less infectious virus is produced in the chronically infected cultures as compared to non-adapted virus grown in equal numbers of C-13 cells. Whereas the inclusions seen in the RE cells were small and medium-sized, the antigen in the infected C-13 cells appeared in the form of large, amorphous cytoplasmic inclusions, described elsewhere (1).

Identity and Properties of the RE-CVS.—Sonicated extracts of RE-CVS cells at the 42nd passage level were mixed with equal volumes of undiluted normal and antirabies sera, as shown in table I. These mixtures were incubated for 1 hour at 25°C . and applied to monolayers of C-13 cells which were stained with FA after 7 days of incubation. The results indicate that the three samples of antirabies sera inhibited the formation of rabies antigen in C-13 cells (table I).

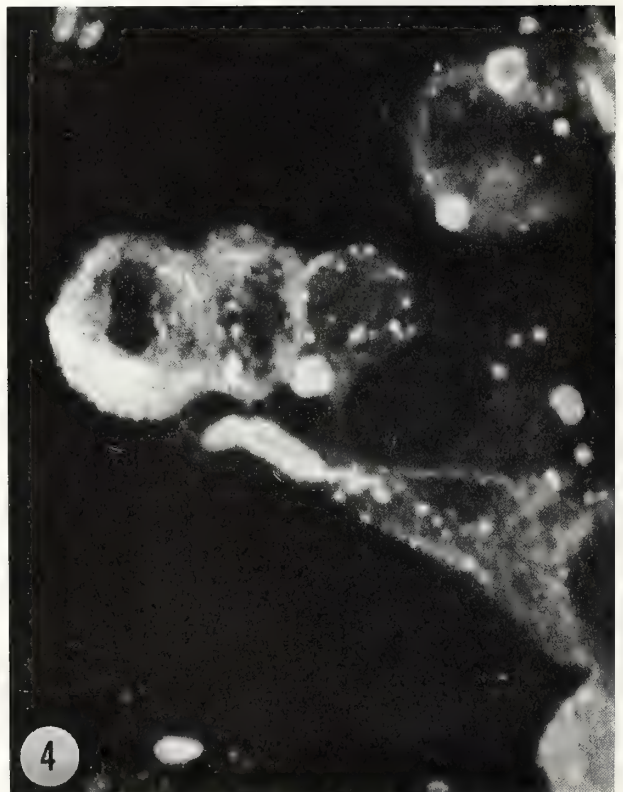


FIGURE 4.—Anaphase $\times 1360$.

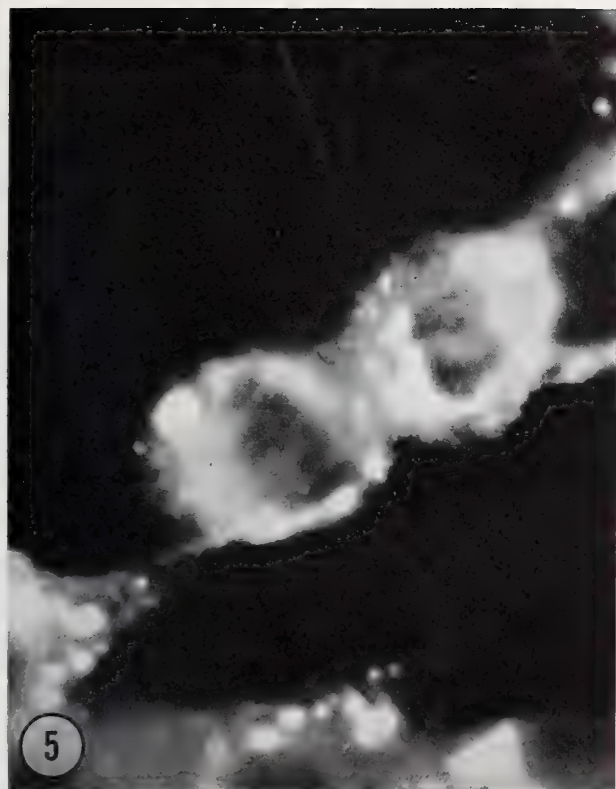


FIGURE 5.—Telophase $\times 1360$. See figure 2.

TABLE I.—Identification of CVS in the RE-CVS¹ Culture

Serum	Species	Percent of C-13 cells showing FA 7 days after exposure to virus-serum mixtures
Antirabies	Human W	0
	Human F	0
	Horse P	0
Normal	Human	50
	Calf	75

¹ Extract of sonicated cells mixed in equal parts with undiluted serum and the mixture incubated for 1 hour at 25° C.

Although the MB-CVS is virulent for adult mice, the results of intracerebral inoculation of mice with virus obtained from RE-CVS cultures indicated gradual loss of virulence for mice, even at an early passage level (figs. 8 and 9). At the 30th cell transfer level, the mortality rate of adult mice injected with sonicated cell extracts obtained from RE-CVS was very low (fig. 9); the same material was only slightly more virulent when injected into newborn mice. Finally, from the 42nd subculture or later, neither adult nor newborn mice died after intracerebral inoculation of RE-CVS cell extracts. Re-

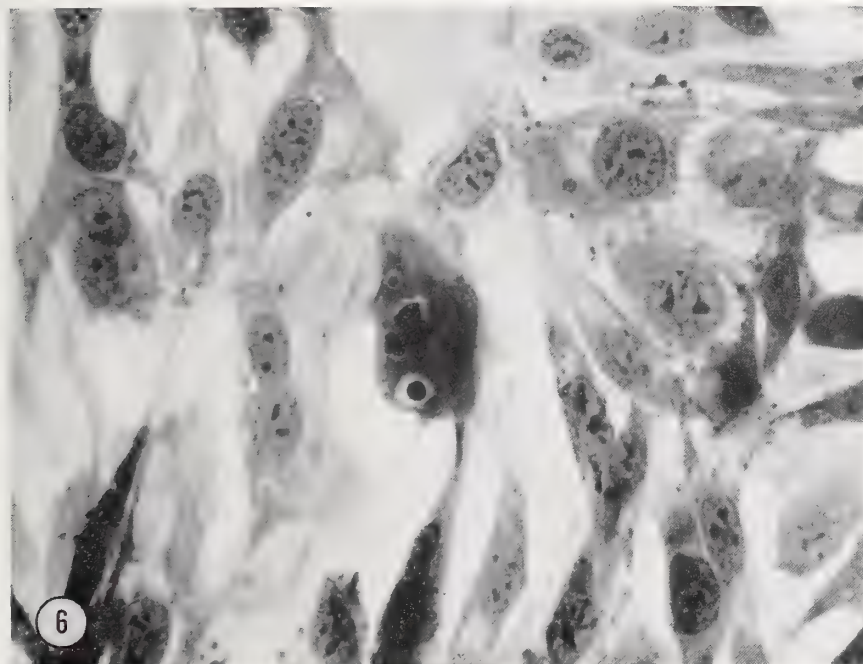


FIGURE 6.—87th subculture of RE-CVS cells. Note the presence of round or oval-shaped intracytoplasmic inclusions surrounded by a clear halo. May-Grünwald Giemsa staining. $\times 740$.



FIGURE 7.—38th subculture of RE-CVS cells. Arrow indicates the oval, uniformly dense inclusions. Phase contrast microscopy. $\times 1200$.

sults of intracerebral challenge with MB-CVS indicated that mice injected originally through 10^{-2} dilutions of the RE-CVS cell extract were protected. These extracts were still infectious for tissue culture systems at 10^{-3} or 10^{-4} dilutions (fig. 9).

Newborn and young adult rabbits injected intracerebrally with 0.05 ml. of a sonicated extract of RE-CVS cells at the 54th subculture level and observed for 5 weeks showed no signs of illness.

To test the antigenicity of the virus passed in RE-CVS cells, sonicated cell extracts suspended in their own tissue culture medium were injected intraperitoneally into adult mice. These mice were challenged 21 days later with MB-CVS either by the intracerebral or intramuscular route (table II). Immunized mice withstood the challenge better than the control mice (table II), but the antigenicity of the RE-CVS, as determined by this mouse protection test, was not very high.

Interference.—From the obtained data, it was apparent that virus antigen is carried in all RE-CVS cells and relatively small amounts of infectious virus are produced by these cells (fig. 8). The RE-CVS system was, therefore, tested for susceptibility to superinfection with homologous and heterologous virus.

As shown in table III, RE-CVS and RE cultures were exposed to infection with undiluted MB-CVS and RB-PM virus which remain pathogenic for mice

TABLE II.—Antigenicity of RE-CVS Preparation¹ Injected Intraperitoneally² into Mice

Route of challenge	Results of challenge with MB-CVS			
	RE-CVS immunized mice		Controls	
	LD ₅₀	MR	LD ₅₀	MR
Intracerebral	10 ^{4.9}		10 ^{6.5}	
Intramuscular		8/30		21/30

MR=Mortality Ratio.

¹ Extract of sonicated RE-CVS cells at the 45th subculture level which contained 10^4 TCID₅₀, as determined by titration on C-13.

² One intraperitoneal inoculation followed by challenge with MB-CVS 21 days later.

TABLE III.—Resistance of RE-CVS to Superinfection with Two Strains of Rabies Virus Pathogenic for Mice

Tissue culture	LD ₅₀ titer in mice inoculated with medium obtained from:		
	Noninfected cultures	PM-infected cultures	CVS-infected cultures
RE-CVS	<Undiluted	<Undiluted	<Undiluted
RE	0	10 ^{3.5}	>10 ^{5.5}

Titers of viruses grown in RE-CVS and RE cells were tested 6 days after infection with PM and CVS.

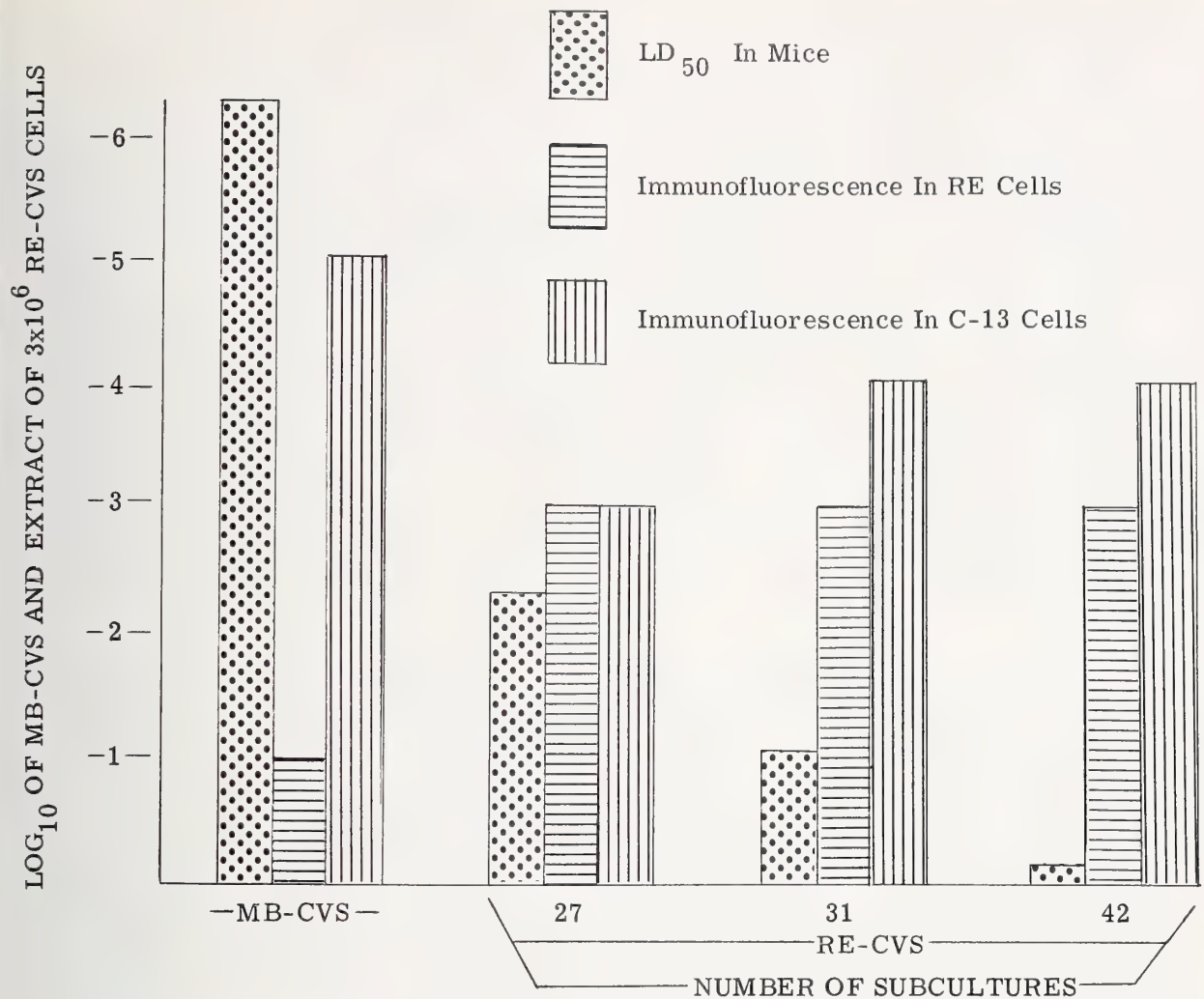


FIGURE 8.—Infectivity of MB-CVS and RE-CVS cell extract for tissue culture systems and mice.

after several passages in the RE tissue culture system (see above). Following 6 days' incubation, the culture media were titrated in mice injected intracerebrally. Whereas the LD₅₀ titer of medium from the control RE culture infected with MB-CVS and RB-PM was 10^{3.5} and >10^{5.5}, respectively, not a single animal died after inoculation with the undiluted medium obtained from RE-CVS cells superinfected with MB-CVS and RB-PM. Thus, growth of the RB-PM virus and MB-CVS was inhibited in the RE-CVS cells. Similar results were obtained when monolayers of RE-CVS and RE cultures were exposed to infection with heterologous VSV (table IV) since RE-CVS inhibited multiplication of at least 7 × 10³ or more PFU of VSV. These experiments demonstrate the capacity of RE-CVS cells to exclude replication not only of other closely related strains of rabies virus,

TABLE IV.—Plaquing Efficiency of VSV in RE-CVS and RE Tissue Culture Systems

Tissue culture	Number of plaques of VSV per plate of culture exposed to virus dilutions (Log ₁₀)				
	3	4	5	6	7
RE-CVS	0	0	0	0	0
RE	Confluent		37	7	0

VSV = Vesicular stomatitis virus.

but also unrelated VSV. In this respect, the RE-CVS system seems to differ from carrier cultures of polyoma L cells (11-13) and mumps in human conjunctiva cells (14) where interference was only observed against closely related viruses.

Repeated attempts to isolate an interferon-like substance from the RE-CVS cell system were unsuccessful.

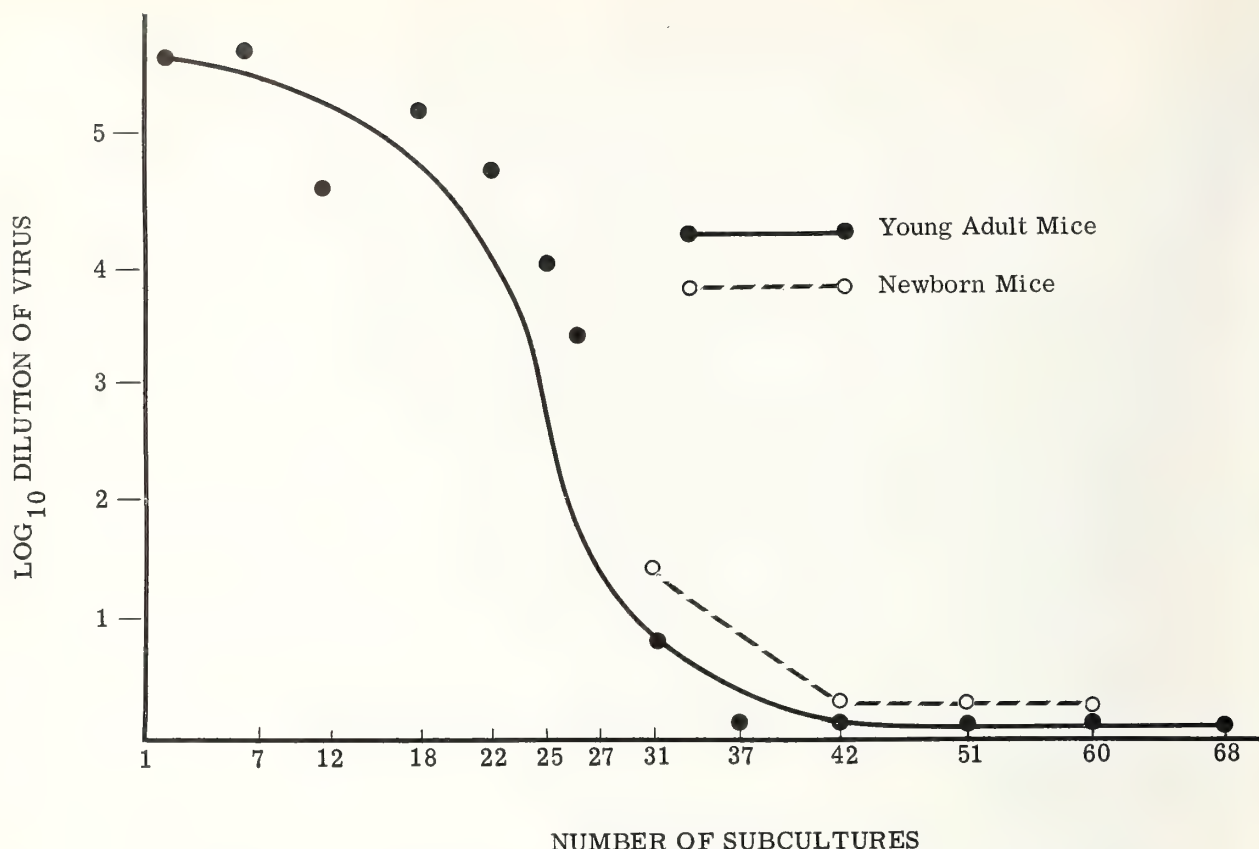


FIGURE 9.—Loss of pathogenicity of re-adapted CVS for mice injected intracerebrally.

Comparative Characteristics of the RE-CVS and RE Cell Lines.—Except for the intracytoplasmic inclusions which could be observed in RE-CVS cells under phase contrast or after staining, no differences could be detected between cells of the RE and RE-CVS series. Cells of both RE and RE-CVS cultures have an elongated epithelioid morphology; they exhibited the same growth rate when subcultured twice a week, and both demonstrated the contact inhibition phenomenon upon formation of a confluent monolayer.

The plating efficiency of the cells in the two systems was determined by plating 500 cells of each tissue culture line in 50 mm. Petri dishes, incubating them for 16 days, and then counting the number of colonies. The plating efficiency of the two cell lines was found to be very similar (table V). There was no indication that the presence of rabies virus interfered with the viability of the RE-CVS cell system.

DNA and RNA Labeling Experiments.—The experiments dealing with the incorporation of labeled nucleic acid precursors failed to reveal any difference between the RE and RE-CVS cells. Thus, after 30 minutes of incubation with H₃-thymidine, the propor-

TABLE V.—Comparative Plating Efficiency of RE-CVS and Noninfected RE Cultures

Number of cells plated	Culture	Experiment	Number of colonies	Percent plating efficiency
500	RE-CVS	1	11	2.1
		2	14	2.8
	RE	1	16	3.2
		2	9	1.8

tion of labeled nuclei was 14.8 percent for the RE and 15.7 percent for the RE-CVS cells. Given an approximate DNA synthesis time of 8 hours (15), it can be calculated that the generation time of both cell types is 50 hours. Even if H₃-thymidine was kept in the medium for as long as 7 hours, no cytoplasmic labeling occurred.

When H₃-uridine was used, the pattern of nuclear and cytoplasmic labeling in the two cell cultures was identical. The number of grains over the inclusions in the RE-CVS cells did not differ from other cytoplasmic zones. When the labeled precursor was pres-

ent in the medium for 28 hours, no accumulation of RNase-resistant RNA was observed anywhere in the cells. These findings indicate that the green staining of the inclusions by acridine orange previously reported (3) is not due either to the presence of DNA or of double-stranded RNA.

Chromosomes of the Two Lines.—Although RE cultures are not strictly diploid, the karyotype of the RE-CVS cells in the 54th passage cells was examined to determine the effect of the virus infection on the chromosomes of its carrier cell.

Preliminary examination of chromosomes of 20 cells of the RE-CVS cell strain (91st passage) showed pseudodiploidy involving monosomy for one of the smallest chromosomes. Most of the counts of RE-CVS were near diploid, only four cells were exactly 44 (2n) and of these cells, three were distinctly abnormal in chromosome morphology. However, examination of only 10 RE cells showed 3 instances of monosomy for 1 of the smallest chromosomes in 3 pseudodiploid cells, minute chromosomes, "true" chromatid breaks, and a tri-radiate configuration. The tetraploid level was increased from 7 percent in the RE to 25 percent in the RE-CVS cells. Because of these chromosomal aberrations in the noninfected RE culture, indicating instability, it was not possible to draw any conclusions as to the effect of the virus infection on the karyotype.

Effect of Antirabies Serum.—Several workers (11, 13, 14) have described how chronically infected cultures can be cured by transferring cells in the presence of specific antiviral serum. Similar studies were conducted with the RE-CVS culture after testing the effect of antirabies serum on the spread of extracellular virus.

Human antirabies serum (Human F) was inactivated for 30 minutes at 56° C. and incorporated at a 20 percent concentration into the tissue culture medium. For control purposes, normal medium and medium with added inactivated normal human serum were used. In each case 2×10^5 RE-CVS cells were mixed in suspension with 2×10^6 RE cells and the mixture plated out on coverslips in Petri dishes (table VI). All the plates were then incubated for 6 days, at which time the cells were stained by FA and May-Grünwald Giemsa. The results, shown in table VI, indicate that the presence of antirabies serum in the tissue culture medium prevented, to a large degree, the spread of extracellular virus from the RE-CVS cultures to the RE cells.

Following this experiment, an RE-CVS culture, starting from the 51st subculture passage, was main-

TABLE VI.—Prevention of Spread of Infective Virus from CVS-Infected RE Cells to Fresh RE Cells by Treatment with Antirabies Serum

Serum ¹ in medium	Exp.	Percent of cells 6 days after ex- plantation of a mixture ² of RE-CVS and RE cultures showing:	
		FA	Inclusion bodies (May-Grünwald Giemsa)
Anti-rabies ³ (human)	1	20	20
	2	15	10
Normal (human)	1	100	70
	2	100	75
Normal (calf)	1	100	75
	2	100	75

¹ At 20 percent concentration.

² 2×10^5 cells of CVS-infected RE culture mixed with 2×10^6 cells of noninfected RE culture and explanted in cultures.

³ Human F serum inactivated for 30 min. at 56° C.

tained in immune-serum medium (Human F) for nine consecutive cell transfers for a total of 53 days of cultivation. Even at the end of this period, 100 percent of the RE-CVS cells showed the presence of inclusions stained by FA. Thus, it appears that while the antirabies serum effectively inhibited the spread of extracellular virus, its presence in culture medium could not interfere with the cell-to-cell transmission of the virus during mitosis.

Inactivated serum had to be used in these experiments since preliminary observations indicated that fresh antirabies serum was cytolytic for the RE-CVS cells. In order to investigate this point further, RE-CVS cells, either in suspension or in monolayer as shown in table VII, were exposed to antirabies serum which was not cytolytic for RE cells (Human F). Complete cytolysis of the RE-CVS cells was observed after exposure to fresh antirabies serum, whereas the same serum did not cause lysis of the same cells after inactivation for 30 minutes at 56° C. Addition of fresh hamster complement to the inactivated serum restored its cytolytic properties. There was no lysis of the RE cells exposed to the same fresh antirabies serum and, conversely, normal noninactivated serum (without rabies antibodies) and complement had no effect on the RE-CVS cells.

Virus Release from Cells of the RE-CVS Cultures.—Since, as shown in figure 8, the extract of one million RE-CVS cells contained about 10^4 infectious virus particles, it seemed improbable that all the cells of the culture appeared to be capable of releasing infectious virus at a given time even though virtually all

TABLE VII.—Cytolytic Effect of Antirabies Serum

Serum	Treatment of serum	Effect of serum on RE and RE-CVS cells			
		Cells in suspension		Cells in monolayer	
		RE-CVS	RE	RE-CVS	RE
Antirabies ¹	Nonin-activated.	++++	0	++++	0
	Inactivated ² plus complement. ³	+++	0	N.t.	N.t.
	Inactivated	0	0	0	0
Normal	Nonin-activated.	0	0	0	0
Complement	Nonin-activated.	0	0	N.t.	N.t.

¹ Human F serum used in 1:4 dilution.² Incubated for 30 min. at 56° C.³ 1:10 dilution of freshly collected hamster serum.

0=No lysis.

++++=Complete lysis with no recovery of viable cells.

+++ =Almost complete lysis but a few cells survived and gave origin to colonies.

N.t.=Not tested.

of the cells showed the presence of rabies virus antigen. In the absence of a reliable plaque system for quantitative determination of virus, difficulties were encountered in deriving an assay for virus release from the individual RE-CVS cell. Finally, the following ad hoc technique was devised for this purpose. (See also Materials and Methods.)

RE-CVS cells at the 84th subculture level were washed three times with PBS and diluted to a concentration of 1,000 cells per 6 ml. Ten Petri dishes containing a monolayer of C-13 cells grown on two coverslips (11 x 22 mm.) were seeded with 6 ml. of

RE-CVS cells per dish and incubated at 37° C. Eighteen hours later, one coverslip from each dish was removed and stained by FA in order to determine the number of dispersed RE-CVS cells which adhered to the C-13 monolayer on the coverslip. The other coverslip was removed after 6 days of incubation and also stained by FA. This time only the centers of aggregations of 10 or more cells were counted. It was assumed that those cells were the C-13 cells infected by the virus released by the dispersed RE-CVS cells.

The results obtained with the coverslips removed after 18 hours and 6 days, respectively, were matched for the same Petri dish (table VIII). With the exception of coverslip 9, there was a fairly uniform count of dispersed RE-CVS cells adhering to the C-13 monolayer. Out of 10 dishes showing the presence of RE-CVS cells, 3 failed to show fluorescing centers after six days of incubation, and in the remaining 7 the number of fluorescing aggregates of presumably C-13 cells varied from 1 to 4 per dish. Taking into consideration the average values, about 4 to 5 percent of the RE-CVS cells were releasing infectious rabies virus at a given time of cultivation.

To eliminate the possibility that aggregates of fluorescing cells, observed 6 days after the RE-CVS cells were seeded, represent the multiplying RE-CVS cells and not the fluorescing C-13 feeder layer cells, the experiment described above was repeated with a modification: C-13 monolayers were grown on three coverslips per each of the five Petri dishes and, at 18 hours after seeding with RE-CVS cells when one coverslip was removed for staining, coverslips in three dishes were exposed to fresh antirabies serum (Human F) at a 1:4 concentration. Two Petri dishes were left without exposure to antirabies serum. Five hours later one coverslip from each of the five Petri dishes was removed for FA and the five dishes, still containing one coverslip each, were washed three times with PBS

TABLE VIII.—Assay for Presence of Virus-Yielding Cells in RE-CVS

Time after seeding	Number of cells or cell aggregates showing FA										
	Coverslip No.										Average
	1	2	3	4	5	6	7	8	9	10	
18 hrs. ¹	26	35	46	39	42	31	33	44	12	39	34.7
6 days ²	0	3	0	1	3	1	4	2	0	1	1.5

¹ Dispersed RE-CVS cells.² Aggregates of not less than 10 C-13 cells.

and reincubated until the 6th day after seeding when the remaining coverslips were stained with FA.

The results of the experiment, shown in table IX, indicate that 5 hours after treatment with antirabies

TABLE IX.—Assays for Virus-Yielding Cells in RE-CVS in Presence of Antirabies Serum

Petri Dish	Treatment	Number of cells or cell aggregates showing FA at time after seeding					
		18 hrs.		23 hrs.		6 days	
		No. ¹	Average	No.	No. ²	Average	
1	With anti-rabies serum	103	76	0	2	4	
2		92		2	6		
3		34		0	4		
4	Without anti-rabies serum	61	68	81	4	3	
5		74		53	2		

¹ Dispersed cells.

² Aggregates of not less than 10 cells.

serum (23 hours after seeding) cells showing fluorescence at the 18th hour after seeding, were virtually eliminated. However, the number of colonies showing FA on the 6th day after seeding was essentially identical in cultures treated with antirabies serum and in those receiving no treatment at all. These results suggest that the fluorescence of cell aggregates observed on the 6th day after seeding with the RE-CVS cells was indeed that of the C-13 cells which became infected by the contact with RE-CVS cells during the 18-hour incubation period.

DISCUSSION

The results of the present study indicate that rabies virus can infect mammalian cells in culture and that it can propagate in the infected cells for a prolonged period of time, possibly indefinitely, without interfering with the mechanism of cellular replication. In this respect rabbit endothelial (RE) cells have the advantage over other cell systems infected with rabies virus (1, 2) because the virus remains in a true endosymbiotic state possibly in relation to every single cell in the culture. In systems such as human diploid cell strains and neonatal hamster kidney fibroblasts, the intracellular events following infection lead to the formation of large inclusion bodies which interfere with the mitotic process and ultimately cause degeneration of the culture (1, 2). No such phenomena were observed in the RE-CVS system. Cells, once infected, formed small to medium-sized inclusions, demonstrated either by FA or by histological means

(3) and these inclusions did not seem to interfere with the mitotic process of the RE-CVS cells. Indeed, the rabies-infected cultures (RE-CVS) have now been maintained for more than 93 cell transfers.

The term "endosymbiotic" has been used to describe the relationship between the rabies virus and the RE-CVS system since the more commonly used term "carrier" seemed to be reserved for a situation where only a fraction of the cell population at a given time is infected, and the virus constantly released in small amounts by these cells often infects other cells of the same culture. In the carrier system, the infected cells probably lyse continually, but their lysis escapes detection among the large fraction of noninfected, normally dividing cells (13).

There have been several reports of culture systems in which cells containing viral material undergo what appears to be a normal mitotic cycle (16-21). However, the RE-CVS system has been carried through more transfers than any other culture except for L cells infected with polyoma virus. In the latter case, the majority of the cells show the presence of viral antigen in the cytoplasm as in the RE-CVS system. However, in contrast to the RE-CVS cells, a small percentage of L cells infected with polyoma virus (a DNA virus) also show nuclear fluorescent staining. It has been postulated that these cells produce infectious virus (11) and lyse, and that, in other L cells, the presence of viral antigen in the cytoplasm could possibly be due to phagocytosed virus in the otherwise healthy cells.

Although all cells of the RE-CVS cultures showed the presence of viral antigen in the cytoplasm, it must still be determined why only 4 to 5 percent release infectious virus at any given time and how these cells differ from other cells. Inquiry into this problem may be facilitated by finding methods which inhibit mitosis of RE-CVS cells and increase the virus yield.

Another important difference between the "endosymbiotic" infection and the "carrier" systems is that treatment with antiviral serum did not "cure" the virus infection in the RE-CVS cells during 9 cell transfers in the course of 53 days. In contrast, polyoma L cells were "cured" after exposure to antipolyoma serum for 81 days of cultivation (12).

The RE-CVS cells showed a resistance to infection with homologous as well as heterologous viruses in the absence of an interferon-like substance. Interferon could not be demonstrated in the polyoma L cell system (12) but in this case, in contrast to the RE-CVS cultures, interference was restricted to closely related viruses. Interference against closely

related virus was also observed in another "carrier" system of human cell cultures chronically infected with mumps virus (14). The fact that all the cells of the RE-CVS system are infected by the rabies virus may in itself account for the resistance to infection by other viruses. This would eliminate the need for a transferable resistance factor produced by the infected cells and released into the culture medium.

The properties of the CVS became modified while the CVS was endosymbiotic with the RE cells. During the course of 40 passages, the virus lost not only its virulence for mice, but also, to a large extent, the ability to multiply in mice *in vivo* as indicated by the relatively poor antigenicity of the RE-CVS for mice. No pathogenicity for rabbits was observed after the passage of CVS virus in RE cultures.

Finally, it should be pointed out that the sites at which the rabies virus persists in the body of an animal or man during the prolonged incubation period between exposure and illness are still unknown. Similarly, the mechanism through which the virus is capable of maintaining itself in such a latent state remains a mystery. It is perhaps possible that a parallel may be found between the endosymbiotic relationship of the rabies virus to the RE-CVS cell and its prolonged persistence in cells of the whole organism without causing injury to the infected cells or to the host. What remains to be explained is the mechanism through which this endosymbiotic relationship is disrupted, possibly leading to the production of large quantities of virus which ultimately destroy the host.

SUMMARY

RE cultures infected with a fixed rabies virus were studied. The virus can propagate itself in these cells for an indefinite period of time without interfering with cell growth. The present study characterizes this truly endosymbiotic relationship.

Virus-specific antigen was detected in the cytoplasm of each cell by fluorescein-labeled antirabies serum but only 4 to 5 percent of the cells released infectious virus. All cells undergoing division showed viral antigen throughout the mitotic process. Also, the growth rate, plating efficiency, and morphological characteristics of both the infected and control cultures were identical. No difference was detected between the RE and the RE-CVS cell populations by RNA and DNA labeling experiments, using H_3 -thymidine, and H_3 -uridine. Although antirabies serum effectively inhibited the spread of extracellular virus, it did not interfere with cell-to-cell transmission of the virus during mitosis, in the course of 9 cell transfers during 53 days. RE-CVS

cells, when exposed to fresh antirabies serum, lysed completely but inactivated serum had no lytic effect. The addition of fresh hamster complement to the inactivated serum restored its cytolytic properties.

The serially-passaged RE-CVS virus gradually became less virulent for mice and displayed a weak antigenicity in the mouse protection test.

Another feature of the RE-CVS cell system is its resistance to infection with homologous and heterologous viruses despite the apparent absence of an interferon-like substance.

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DISCUSSION

PATERSON: Is the lysis of the cell geared to an action between antibody and part of the intact virus, part of the cell membrane, or could it be an interaction between antibody and, let us say, a viral fragment of an unattached type in your tissue culture system? In other words, could you have a breakdown of virus which would leak out of the cell and lead to an interaction between the antibody you added and the fragment, which rubs up against other intact cells and lyses them, even though they themselves are not infected?

KOPROWSKI: That question is difficult to answer. I suspect that there is an interaction between the

serum and viral antigen present either near cell membranes or spreading over cell membranes.

PATERSON: This is an important point because if it is what you say, it would suggest that wherever you see a cell plaque, then that cell is infected with virus. If it is the other way, you get the same effect by actually, in reality, having very few cells that might be infected with the virus, and from the standpoint of latency that might be quite a different thing.

KOPROWSKI: Results of fluorescent antibody staining indicate that all cells in culture contain the rabies virus and that 4 to 5 percent of these cells produce infective virus. In spite of the fact that rabies virus is an RNA virus growing in the cytoplasm, the endosymbiotic relationship resembles lysogeny more than with any other animal virus. If we could "induce" cells showing presence of rabies antigen to produce a large quantity of infective virus, then the similarity to the lysogenic system would be complete. So far only irradiation of the cultures increases the yield of infective virus. What we are studying now are factors which may precipitate "frank illness" in the body of an animal exposed to rabies "ages ago."

HOTCHIN: It seems to me that you do have a very good claim to a lysogenic animal virus system here. I wonder what relevance you think this might have by analogy in the slow virus situation. What bearing might this system have on the slow virus concept with respect to late onset of something like an autoimmune disease consequent upon a transformation of a type like the tumor transformation?

KOPROWSKI: I feel that perhaps a parallel may be drawn between the endosymbiotic relationship observed for rabies virus in a tissue culture system and the fate of a "slow virus" (CHINA or chronic infectious neuropathic agent) during the extended incubation period in the body of the animal. You may even postulate, stretching the parallel to the limit of an absurd hypothesis, that if the level of virus specific antibodies reaches a certain threshold in the animal body, cells infected with a CHINA may lyse, release infective virus and the animal will become sick and die. In the case of rabies this hypothesis could be easily disproven, if mice surviving street virus infection were given an enormous dose of antirabies serum without effect.

CASALS: One problem is that when bats, as you know, are infected with rabies and become carriers for months and months, they never show any signs or symptoms but nevertheless continue to put out infectious virus.

Chronic State of Rubella

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It may appear somewhat surprising to be discussing rubella as a chronic infection. This disease is known as "3-day measles" or "little measles," an illness of short duration and mild intensity. It is considered of minimal significance except when infection occurs early in pregnancy, in which case, congenital malformations occur in 18 percent of the children. Recent findings, however, indicate that with the infection of the mother early in pregnancy, the fetus may become "chronically" infected and this infection persists through birth and for a number of months in infancy. In addition, at birth the child may have the clinical syndrome which we have called "Acute Congenital Rubella of the Newborn."

In the present report, I will review some of the recent information concerning rubella, the congenital rubella syndrome, the chronic state of rubella, and acute congenital rubella of the newborn.

Rubella in Children and Adults

With the development of techniques for the isolation and cultivation of rubella virus and the detection of antibody, a considerable amount of information has been obtained concerning natural infection in children and adults. One of our studies was conducted during the epidemic of rubella on St. Paul Islands in the Pribilofs, Alaska, in June 1963 (1). Intensive clinical and laboratory observations were obtained for 23 male and 23 female children under 19 years of age for a 2-week period. The children were examined and throat swab specimens were obtained daily. Serum specimens were taken from each patient at the onset and termination of the intensive study period and 3 months later.

Of the 46 patients tested, approximately 60 percent developed rash and posterior auricular or occipital

nodes, 40 percent had nodes only, and one had no clinical findings. All of the patients experienced infection with rubella as evidenced by the development of neutralizing antibody during the period of the study. The clinical findings did not differ between the two sexes.

For the patients with rash and nodes, antibody was first detected the day after the onset of rash; and was present in all samples obtained on subsequent days. Virus was isolated from the nasopharynx as early as 13 days before rash; the majority of patients had positive isolations 5 days before rash, and all individuals tested had virus 2 days before the onset of rash. Virus persisted in the nasopharynx, following rash, for at least 2 days, and in some instances was still present 6 days later. Adenopathy occurred as early as 3 weeks before rash; however, the majority of patients had the onset of nodes 1 to 2 weeks before rash and all patients had adenopathy by the day before rash.

For the patients with nodes only, antibody was first detected 5 days after the appearance of nodes; however, some patients did not have antibody as late as 12 days after the onset of nodes. Virus first appeared in the nasopharynx of these patients either before or after the onset of nodes, and disappeared either before or after the onset of nodes.

It was not possible to determine the total duration of persistence of virus in the nasopharynx in all cases. However, virus was detected in throat specimens for as long as 9 days in individuals with rash and nodes and 4 days for patients with nodes only. Virus was isolated from 80 percent of patients with rash and nodes, and 50 percent of individuals with nodes only. Generally, rubella virus was detected in the first tissue culture passage of throat specimens from patients with rash and nodes while repeated tissue passages were necessary

to reveal virus in similar specimens from patients with nodes only.

These findings indicate that: (1) Rubella may occur without rash in a significant proportion of children; (2) the appearance of posterior auricular or occipital adenopathy may be of assistance in diagnosing rubella with or without rash; (3) children with rubella may be infectious for several days before and after the appearance of clinical disease: attempts to prevent the transmission of rubella may be difficult for this reason; (4) for laboratory confirmation of rubella with rash, throat swab specimens for virus isolation should be obtained at or near the onset of rash: additional data can be provided by antibody determinations with paired serum specimens when the first specimen is obtained near the onset of symptoms; (5) rubella with nodes only may be difficult to confirm by virus isolation studies with throat swab specimens: this form of the disease is probably best established with serological studies utilizing paired serum specimens, the first specimen being taken near the onset of symptoms or at the time of known exposure; (6) rubella with rash appears to produce more severe infection of the nasopharynx than rubella with nodes only.

Congenital Rubella Syndrome

In 1941, Gregg (2) first reported the association between maternal rubella in the first trimester of pregnancy and congenital defects. A number of investigators since that time have shown that a wide spectrum of congenital defects can occur (3, 4, 5). These defects are known collectively as the "Congenital Rubella Syndrome." The most frequent defects are those of the eye, including chorioretinitis, cataracts and corneal cloudiness; congenital heart disease, the most frequent of which is patent ductus arteriosus; dental abnormalities; microcephaly; and mental retardation. The frequency of defects is greatest when rubella occurs in the first month (50 percent), lowest in the third and fourth month (2 to 6 percent) of pregnancy. The types of defects which occur tend to differ with the month of gestation during which infection takes place.

The "Chronic State of Rubella" or Chronic Rubella Infection of the Fetus-Infant

Seltzer first reported the recovery of rubella virus from the fetus of a mother who had rubella during the first trimester of pregnancy (6). In our studies, virus

was isolated from 60 percent of 25 fetuses which were obtained by therapeutic abortion following maternal infection with rubella (7). In one case, the virus was recovered from almost every organ of a fetus 77 days after maternal infection. At the same time, the mother had a high titer of rubella neutralizing antibody in her blood.

Additional studies of the isolation of virus from fetuses have been reported by Heggie (8) and Alford et al. (9). Furthermore, Alford et al. (9) recently showed that virus could be isolated from several children with congenital rubella who were 1 to 4½ months old. In two of their cases, there was thrombocytopenia and moderate elevation of bilirubin. In our studies, virus could not be recovered from any of 12 institutionalized children with rubella syndrome defects who were 6 to 14 years of age (10). This suggests that the "chronic state of rubella" is probably limited to early childhood.

"Acute Congenital Rubella of the Newborn"

The isolation of rubella virus from children with congenital rubella has been confirmed in our own laboratory (10) and the syndrome "acute congenital rubella of the newborn" has been recognized frequently throughout this country following the 1964 epidemic of rubella. This syndrome appears at birth and includes primarily thrombocytopenia, enlargement of the liver, spleen, and bleeding tendencies. In some instances, there is elevation of the bilirubin and fatal termination. The syndrome has been recognized previously in children with rubella syndrome defects (11); however, preliminary evidence suggests that it occurred frequently in this epidemic and without congenital rubella syndrome defects which can be detected in the newborn.

In conclusion, I would emphasize that the findings for rubella should alert us to the fact that in considering viruses as possible causes of diseases of unknown etiology such as multiple sclerosis and amyotrophic lateral sclerosis, we must include a thorough consideration of infectious agents which are already known and have been studied in the laboratory. The recent studies of chronic rubella infections and the production of tumors in experimental animals with adenoviruses and SV 40 indicate that we must consider the possibility of a wider scope of clinical effect than formerly recognized for some of these agents.

Rubella produces an acute, limited disease in children and adults. When infection occurs in the first trimester of pregnancy there is an 18 percent incidence of children with congenital malformations. In addition, a "Chronic State of Rubella" occurs in the fetuses and infants of women infected during pregnancy. The infection of the child persists through birth and for the first months of infancy, but infection is not present in older children. "Acute Congenital Rubella of the Newborn" has been recognized frequently in the 1964 epidemic. The principal findings are thrombocytopenia, enlarged liver and spleen, and in some instances, elevation of bilirubin and fatal termination. These new findings for rubella emphasize our limited knowledge of the effects of known viruses and the need to include consideration of these viruses in studies of diseases of unknown etiology.

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DISCUSSION

- BROWN: What levels of antibody and virus did you find over the first year postnatally?
- SEVER: The antibody levels are comparable to those which we find in the adult, that is, neutralizing titers in our laboratory from 16 to 64. Regarding the virus titer it is hard to titer virus in the throat with accuracy from a throat swab. We have tried to quantitate this in the acute phase, but it is only a rough estimation of the amount of virus present. In the disease in older children, the amount of virus present would seem, in general, to increase and then decrease and is at its highest point during the period of rash; as soon as antibody appears, then no virus can be detected.
- JOHNSON: What about the infants who did not have malformations, but whose mothers had rubella in pregnancy? If they have the rubella virus and not the antibody, then that would be a most intriguing possibility.
- SEVER: We have some patients from whom we were able to recover virus from the placenta, and it seems out of the throat also, who look normal now, but we cannot be sure; for defects like hearing and mild mental retardation it will certainly take some time to be quite sure that the child is not clinically involved. But in the end there might be quite a few from whom we could get virus and who prove to be completely normal.
- MORRIS: Have you prepared cell cultures successfully from these embryos? If so, do the cell cultures carry the rubella virus and, if they do, how do you recognize it?
- SEVER: This has been reported by several groups now who have prepared cell cultures and have obtained CPE's; in fact, there have been people who have been working with human embryonic cell cultures and had a CPE for a long time and now, when they look back, they find that the mother did indeed have rubella. Several years ago, when this whole thing was started, the CPE which occurs in these explanted cell cultures was found to be similar to that reported by Dr. Weller in the primary amnionic plate, the amnionic culture which he has worked with; this is a difficult thing to recognize well, but certainly it has been done.
- KIBRICK: Do you have data on whether expectant mothers who develop rubella in the second and third trimester may also give rubella to their fetuses?

SEVER: So far, we have been able to recover virus through the fourth month. This correlates with the 2-percent incidence of congenital malformation that may occur with rubella in the fourth month of pregnancy. Later than that we have as yet only suggestive evidence from several cases. At least we can say that for a longer period than the 3 months

we are still getting the virus out of the placenta, but it narrows down very quickly, even from the latter part of the first trimester; and the spectrum of organs that are infected, regardless of when you obtain your fetus, is pretty much dependent on when the infection occurred.

Pathogenesis of Infection with Herpes Simplex Virus with Special Reference to Nervous Tissue¹

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Introduction

The recognition that certain viral disorders of animals (e.g., scrapie, visna, Aleutian mink disease) may be characterized by unusually prolonged incubation periods (1 year or more) has stimulated interest in the role of slow, latent, and temperate viruses as a cause of human illness. The association of these agents with chronic degenerative diseases of the central nervous system in animals suggests that viruses with similar properties may be implicated in analogous disorders in man. Herpes simplex virus shares certain properties with these agents. It can produce subclinical as well as acute infections and may persist in occult form possibly for life, giving rise to exacerbations of disease in response to a wide range of stimuli. This agent has a predilection for nervous tissue and can cause acute encephalitis both in man and animals. In the experimental animal, moreover, it can give rise to subacute and chronic encephalitis or remain latent for prolonged periods in the brain. In man, also, there is suggestive evidence to indicate that it may give rise to certain cases of subacute and chronic encephalitis as well as to exacerbations of neurologic disease. A review of this agent and a consideration of its role in neurologic illness, therefore, appear appropriate for inclusion in this symposium. In this report emphasis will be placed on studies elucidating the pathogenesis of herpetic infections and the effect of such infections on nervous tissue.

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General Comments on Herpes Simplex Virus and Herpetic Infections

The structure, biochemistry and serology of this agent have recently been reviewed (1, 2). The virus ranges from 165 to 180 m μ in size and has a DNA core and protein capsid. It also has an outer lipid-containing envelope derived from host-cell membrane and is, therefore, ether sensitive. Although only one antigenic type is known, antigenic subgroups have been demonstrated. Strain differences have been observed on the basis of varying pathogenicity for different hosts, morphologic effects in tissue culture and quantitative differences in antigenicity (2).

This agent has a predilection for tissues derived from embryonic ectoderm and most commonly attacks the skin, mucous membranes, eye, and central nervous system. Under special circumstances or in special hosts (e.g., severe malnutrition, newborns) a generalized infection, involving also mesodermal tissues, may occur. Primary infection may be either apparent or subclinical. It is acquired chiefly through the mouth and, in adults, by sexual contact as well. Abraded or broken skin at any site may also serve as a portal of entry.

The characteristic lesion in herpetic disease is the vesicle. Primary infection is associated with a specific antibody response and with localization of the agent in occult form at varying sites. Thereafter, despite the presence of neutralizing antibody, various exogenous or endogenous stimuli may induce activation of infective virus at these sites, either with or without the appearance of clustered, vesicular lesions which are characteristic of recurrent herpetic disease. In the

eye, primary and recurrent disease may be manifested by conjunctivitis, keratitis or both. In the central nervous system primary infection may result in encephalitis, meningoencephalitis or aseptic meningitis.

It has recently become apparent that herpetic infection may involve not only the external genitalia in the female, but also deeper genital structures, giving rise to a herpetic vulvovaginitis and cervicitis, as manifestations of either the primary or recurrent disease. In several patients yielding this virus, moreover, genital lesions or symptoms suggestive of herpetic infection were absent, the diagnosis being initially made on the basis of characteristic cytology (multinucleated giant cells with type A inclusions) in cervical smears (3). The frequency with which herpetic infection occurs or recurs in the female genital tract is indicated by the results of a survey of routine Papanicolaou smears. During a 15-month period, cytology characteristic of herpetic infection was found in over 50 cases (4).

Several general considerations may be of interest to those planning to work in the laboratory with materials suspected of containing this agent (2). Specimens which cannot be inoculated immediately after collection, may be stored for long periods at -70°C . without loss of infectivity, or for several hours at refrigerator temperatures (4° to 8°C .). Infected tissues can be preserved for 6 months or more at 4° to 8°C . if stored in 50 percent glycerol. Preservation of viral infectivity in infected fluids is aided by the presence of protein-containing substances such as 10 percent animal serum or 0.5 percent gelatin. Virulence is rapidly lost in physiologic saline solution and may be gone after several hours. As an alternative diluent, sterile distilled water may be used. Heparinized blood should not be employed in attempts to demonstrate viremia with herpes simplex since heparin has an inhibitory effect on this virus in tissue culture (5). Although a wide variety of tissue culture systems are satisfactory for isolation of this agent, including primary human amnion and rabbit renal cells, Rhesus kidney cultures are relatively insusceptible and are not satisfactory. Primary cultures of cynomolgus kidney cells are satisfactory. The susceptibility of HeLa cells to this agent varies with different cell lines (6).

Epidemiology

The incubation period for primary herpetic disease ranges from 2 to 12 days although in most cases it falls between 2 to 7 days (7, 8). Primary infection results in disease in about 10 to 15 percent of cases (9, 10).

Although some estimates range as low as 1 percent or less, mild cases are undoubtedly overlooked. In institutional outbreaks or under unusual conditions such as overcrowding, the predisposition to apparent disease is increased. Under such circumstances the incidence of illness may be as high as 50 percent or more (7, 11, 12).

Circulating antibodies appear following primary infection and tend to attain constant levels (in some cases, only after several exposures to virus). Once such levels are established recurrent episodes of infection with this agent are not generally associated with significant changes in antibody titer (10). Exceptions, however, have been observed (10, 13).

A number of investigators have attempted to determine the incidence of herpetic infection at varying age levels and in differing socioeconomic groups. Both the neutralization and complement fixation tests have been utilized for this purpose. In studies comparing results with both technics a close parallelism has been observed. The neutralization test, however, has been somewhat more sensitive since the titer of complement fixing antibodies tends to drop more rapidly after infection (14). Occasionally, in young children, complement fixing antibodies alone have been observed (15).

Andrewes and Carmichael found antibodies to herpes simplex in 21 of 23 hospitalized patients; of 5 medical students also tested, none had antibodies (16). Burnet and Williams in 1939 reported antibodies in 93 percent of 55 hospitalized adult patients and 60 pediatric and adolescent patients as compared with 59 percent for 22 nongraduate hospital workers and 37 percent for 27 university graduates (17). Buddingh et al. determined the incidence of neutralizing antibodies in 591 patients. In those aged 15 or more over 90 percent had antibodies to this agent (18). Yoshino studied the age distribution, in Tokyo, of neutralizing antibodies among 352 subjects. Of those 10 years or older 74 percent or more had antibodies (15).

Somewhat lower figures for antibody incidence have been recorded by others. Scott et al. found neutralizing antibodies in 60 percent of patients by age 5 and in 63 percent of accompanying adults (9, 10). In another study 68 percent of 59 adult dental patients had complement fixing antibodies to herpes (19). In a survey of 149 nurses aged 18 to 29 years who came from homes of good socioeconomic level, Stern et al. found neutralizing antibodies in only 51 percent (20).

This influence of socioeconomic status on antibody incidence is emphasized by unpublished data from our laboratory. Of 63 adult patients on a ward service 84

percent had neutralizing antibodies. This compares with 62 percent for 225 hospital patients under private care. On the other hand, the incidence of such antibodies among several hundred college students and young graduates was about 30 percent; for 83 young physicians and residents it was 37 percent. The relatively low incidence of antibody in the more educated groups undoubtedly reflects a more rigid adherence to basic sanitary principles. As living standards continue to improve, the number of adult susceptibles should further increase with a concomitant rise in primary infections and clinical disease in this age group.

Both neutralizing and complement fixing antibodies are transferred through the placenta to the infant (10, 15, 18). Neutralizing antibodies thus acquired may persist for 6 months or more. An apparent resistance to herpetic infection in the absence of measurable levels of antibody has been observed in infants between 7 and 14 months. It is probable that this is due to levels of passive antibody so low that they are undetected by the technics employed (21).

Latent Infection

The persistence of herpes simplex virus in latent form following infection and the ability of this agent to emerge repeatedly in the presence of antibody have long been recognized, but the mechanism is still obscure. By analogy with lysogenic bacteria, it seems likely that the genome of this virus becomes incorporated into certain susceptible cells in which little or no complete virus is produced, thus enabling the agent to persist. A hypothesis has been proposed by Herriott to explain the recurrent manifestations of herpetic infection in the presence of antibody. According to this hypothesis inactive viral precursors within the cell are activated by alteration of some control mechanism so that free infective viral DNA is released. Such DNA is unaffected by antibody which neutralizes whole virus, but is susceptible to the action of DNA nucleases which are present in the blood. Under stresses and other triggering mechanisms which precede recurrences of the illness, such as fever and ultraviolet irradiation, nuclease inhibitors, possibly released by white cells, appear, permitting spread of the infectious DNA and occurrence of disease. As the inhibitors are dissipated nucleases reappear, the free infective DNA is eliminated and the infection again becomes latent (22). Against Herriott's hypothesis is a report by Smith that the naked viral particles of herpes simplex, when tested in HEp-2 cells in culture,

are virtually noninfective (23). Wildy and Watson, however, concluded that such particles were infective following tests with a baby hamster kidney cell line (24).

Studies of HeLa cell cultures infected with herpes simplex have demonstrated that virus is not eliminated from such cultures by addition of specific antibody, but instead spreads contiguously from cell to cell producing cytopathogenic changes (25). A similar mechanism of tangential spread may be involved in the development of recurrent lesions in vivo, with circulating antibody contributing to localization of such infections.

Production of interferon in response to herpetic infection has been demonstrated in chick embryo tissue culture (26), in the guinea pig following intraperitoneal inoculation (27), and in the rabbit dermis (28). The observation that rabbits inoculated intradermally with live herpes virus produce interferon at the site of inoculation during the period of maximum viral replication suggests that this substance may also contribute to viral containment both in primary and recurrent herpetic disease (28).

Reactivation

In 1930 Andrewes and Carmichael reported that herpes simplex recurred only in those with neutralizing antibody (16). The first recognition that this infection, once contracted, appeared to persist for life in a unique relationship between virus and host, is attributed to Burnet and Williams (17). Although latency is now a well established property of herpetic infection, the form and site of virus during its quiescent phase and the processes involved in its emergence are not clear.

Experimental studies on reactivation of herpes simplex infections appear to have been confined to rabbits. Perdrau in 1938 observed a spontaneous acute encephalitis in rabbits with onset 6 months or more after they had recovered from an intratesticular inoculation of live herpes simplex virus (29). Subsequent cerebral passage of the affected rabbit brains to uninfected rabbits produced an acute herpetic encephalitis.

Good and Campbell in 1945 showed in rabbits that induction of histamine shock, followed immediately thereafter by an intravenous dose of live herpes simplex virus, resulted in an increased susceptibility to development of herpetic encephalitis. One rabbit, inoculated intramuscularly and again intracerebrally with this virus, with no apparent infection, when sensitized to

egg white 6 months later, developed encephalitis following the induction of anaphylactic shock (30). In subsequent experiments the effect of anaphylactic shock on rabbits previously infected with herpes simplex virus was further investigated (31). Rabbits were sensitized to egg white, then immunized with infective virus intramuscularly. After 1 to 3 months, those who had completely recovered and those who had never shown signs of infection (a total of 44) were subjected to anaphylactic shock. Proved herpes simplex encephalitis was precipitated 19 times in 16 rabbits after incubation periods ranging from 2 to 14 days (mean of 6 days). Virus was isolated not only from the central nervous system of rabbits with anaphylactically induced encephalitis but also from rabbits during periods of quiescence, during chronic encephalitis, and during spontaneous exacerbations of encephalitis. Little correlation was observed between the severity of the shock and the incidence of activation of latent infection. More severe and widespread evidence of the disease was frequently noted during exacerbations than during the initial infections, suggesting that the affected area had enlarged. They concluded that the reactivation was due to a renewal of the initial infectious process.

Attempts to activate latent herpetic encephalitis by chemical means were reported by Schmidt and Rasmussen (32). Rabbits were inoculated intramuscularly with live herpes virus and given live virus intracerebrally after one month. Three categories of disease were observed: (1) encephalitis and death, (2) encephalitis with recovery, and (3) (in 60 percent) no apparent illness. Adrenalin, pyromen, cortisone or glutathione were then tested in rabbits of categories (2) and (3) for their ability to precipitate latent herpetic encephalitis. Of 10 rabbits receiving intramuscular adrenalin 24 to 160 days after their intracerebral challenge, 6 developed fatal herpetic encephalitis with onset within 3 to 8 days. Herpes virus was also detected in the brains of 6 of 8 control animals which had remained asymptomatic after similar immunization and challenge but had received no adrenalin, thus confirming the ability of virus to persist in the central nervous system in the latent state. No effect was observed with pyromen, cortisone or glutathione. It was suggested that local anoxia induced by adrenalin might be responsible for reactivation of the herpetic disease.

Anderson, Margruder and Kilbourne reported that herpes simplex virus in healed rabbit corneal lesions could be reactivated by induction of a corneal Arthus reaction (33). Herpes simplex virus was inoculated into corneas of eight rabbits and the resulting keratitis

was permitted to heal. Six to 9 weeks later the animals were sensitized to horse serum by a series of four to five subcutaneous and intradermal injections. A corneal Arthus reaction was then induced in the left eyes by an injection of horse serum at the site of corneal scarring. Induction of the Arthus reaction was repeated for two to three times on each rabbit at intervals of 2½ to 6 months. The occurrence of positive virus cultures from the challenged left eye during the 14-day period following an Arthus reaction was taken as evidence of induced reactivation, the other eye serving as control. Reactivation of virus was demonstrated in 7 of 19 attempts at induction, virus release generally persisting for 3 to 7 days. Although spontaneous reactivations were occasionally also observed in both the challenged and control eyes, none occurred in control eyes during the 14-day post-Arthus period.

Laibson and Kibrick observed that intramuscular administration of epinephrine to rabbits well after recovery from primary corneal herpes was followed by reactivation of virus and/or reappearance of herpetic keratitis (34). These effects could be repeatedly induced in the same rabbits by parenteral epinephrine. As in previous studies, the occurrence of spontaneous virus release was intermittently observed. It was of interest that neither spontaneous nor epinephrine-induced reactivation of virus was necessarily accompanied by the development of clinically visible lesions. This system differs from that of Anderson et al. (33) in that reactivation was induced in response to a systemic stimulus rather than by severe, local trauma and thus appears to more closely parallel recurrent herpetic keratitis in man. The accessibility of the rabbit cornea to direct observation makes this an ideal system for study of latency and reactivation in herpetic infections. Such studies are now in progress.

Viral Pathways to the Central Nervous System

Herpes simplex virus has a marked affinity for nervous tissues but the pathways whereby it reaches the nervous system have not been clearly defined.

In 1920 Doerr and Vöchting observed that experimentally induced herpetic keratitis progressed to encephalitis in a certain proportion of their rabbits. Since encephalitis also followed intravenous injection of this agent, they concluded that viral spread to the brain was by the hematogenous route (35). In 1923 Goodpasture and Teague (36) and Marinesco and Draganesco (37), as a result of pathologic studies in rabbits, independently proposed that herpes simplex virus reaches the central nervous system by traversing

the nerves; the former workers advocating that the virus spread within axon cylinders, the latter that it progressed through the neural lymphatic spaces. Since neural lymphatic flow was subsequently shown to be centrifugal (38), the hypothesis that neural lymphatics are the source of herpetic central nervous system infection appears unlikely. Studies by Wright in 1953 suggested that the tissue spaces between nerve fibers might also serve as a pathway for spread (39).

Recent studies by Johnson, using fluorescent antibody technics, elucidated the pattern of central nervous system infection with herpes simplex in suckling mice (40). He found that intracerebral inoculation of virus was followed by its appearance in the cerebrospinal fluid with spread to the meninges and subsequent involvement of both neurons and glial elements in the underlying parenchyma. Extraneural inoculation of virus—intraperitoneal and intranasal—produced central nervous system disease by both hematogenous and neural routes. Following intraperitoneal inoculation, virus multiplied in the viscera and produced viremia with subsequent appearance of random foci of infection about the small cerebral vessels as the initial cerebral involvement. This was the most important route of spread. The possibility was not eliminated that spread to the central nervous system may also occur by a neural route although at a slower rate. Following intranasal inoculation, however, virus gained access to the central nervous system by several routes; either by direct invasion of the subarachnoid space, by hematogenous spread similar to that after intraperitoneal infection, or by infection of cells along the olfactory and trigeminal nerves. When spread was neural, it occurred from centripetal infection of the endoneural cells of the associated peripheral and cranial nerves, without evidence of associated axonal involvement. Once it had reached the brain, virus multiplied with no relation to neural pathways. Evidence that centripetal spread of herpes simplex virus occurs has been provided by Wildy (quoted by Johnson) (40), who titrated nerves after footpad inoculation of mice with this agent.

Both adult and immature mice are susceptible to the development of encephalitis following intracerebral inoculation with herpes simplex virus. Adult mice inoculated by an extraneural route show a relative resistance to development of encephalitis as compared with immature mice. Johnson found that the barrier to spread of extraneural virus in mature mice appeared to reside in the macrophages (41). Although macrophages in vitro of suckling and adult mice could be infected with herpes simplex virus with equal ease, the

suckling mouse macrophages infected other cells in contact with them whereas adult macrophages did not. These observations suggest that macrophages may play an important role in spread of extraneurally inoculated virus to the central nervous system in suckling mice. In the adult host, however, these cells may serve as a barrier to such spread. The nature of this change in the macrophages which develops with age was not determined (41).

Less is known concerning the pathways whereby this virus gains access to the central nervous system in man. Johnson's failure to demonstrate axonal involvement during experimental herpetic infection in the mouse does not necessarily exclude an axonal route. It is possible that virus may exist in an incomplete state within the axon analogous to the eclipse phase of viral replication and may, therefore, not be demonstrable by current technics.

There is indirect evidence suggesting that this agent may infect the central nervous system of man by a hematogenous route as well as by nerves. Such hematogenous spread has been demonstrated in mice (41). It is known that viremia may occur during primary infection in man (9, 42). It may also be pertinent that herpes simplex virus has been shown to multiply in human leukocytes in vitro (43).

Herpes Simplex Encephalitis

Animal Hosts

Acute herpetic encephalitis has been experimentally produced in a wide range of animal hosts including the mouse, rabbit, guinea pig, and hamster. The routes of inoculation have varied depending on both the species and age of the animal (2, 44). Signs of acute encephalitis differ somewhat in different hosts but may include fever, sluggishness, hyperactivity on stimulation, tremors, incoordination, spasticity, convulsions, pulling of the head to one side (prominent in rabbits), paresis, and death.

A number of responses have been described in the rabbit following intramuscular inoculation with this agent (31). Some rabbits remain apparently well; others develop an encephalitis of varying degrees of severity, generally progressing to death over a 3- to 4-week period; a small number have mild disease and appear to recover completely; and a few develop a chronic, ingravescent encephalomyelitis with low grade fever, lethargy, apathy and intermittent exacerbations of more acute disease.

In acute herpetic encephalitis in the rabbit the localization of the lesions in the brain is determined by

the route of viral invasion. The vessels in affected areas show perivascular cuffing, and hypertrophy and multiplication of the lining epithelium. The parenchyma in these areas reveals initially a polymorphonuclear and lymphocytic infiltration. Subsequently, a mononuclear infiltrate, destruction of nerve cells, hemorrhage, and necrosis are evident. The characteristic intranuclear inclusion bodies of herpes simplex may be present in oligodendria and occasionally in nerve cells in the vicinity of necrotic areas. These inclusions are sparse in the terminal stages of encephalitis in rabbits (45). The meninges show, initially, mixed infiltrates; later these are predominantly mononuclear (44).

Essentially similar histopathologic findings have been observed in rabbits with latent herpes simplex encephalitis which succumbed to this disease following activation of the infection by adrenalin. Intranuclear inclusion bodies, however, were not demonstrated (32).

Rabbits with the subacute or chronic form of herpetic encephalitis are of interest since they differ both clinically and histopathologically from those with the acute disease. Pathologic examination of these animals reveals widespread meningeal, perivascular and parenchymatous chronic cellular infiltration; extensive destruction of nerve cells in variable areas of the brain; areas of true and pseudo-calcification and early stages of scar tissue formation. The chronicity of the lesions is most marked in animals which have survived the longest (45). Herpes simplex virus has been recovered from brains of animals with the chronic form of this disease (45).

Man

The prominent clinical features of herpetic encephalitis include headache, drowsiness, and fever. Seizures are common as are focal neurologic signs (reflex asymmetry, Babinski signs, and cortical sensory loss). Meningeal signs may or may not be present. In some cases behavioral changes of a psychologic nature are prominent initial symptoms. A confusional-hallucinatory state is frequently present during the acute phase. This may be followed by drowsiness, stupor or coma. In patients who survive this phase, a period of hyperactivity may develop. Neurologic residua are common, especially those referable to focal necrosis of the orbital and temporal regions, such as anosmia, isolated memory loss and dysgeusia. The intellect, however, may be relatively well preserved. In some patients aphasia, mental retardation and paresis have been observed (46, 47, 48, 49).

Pathologically, herpetic encephalitis in man is most commonly a disease of the cerebral cortex, with progressively less involvement of the central white matter and basilar structures. The lesions tend to be asymmetric and generally affect the medial temporal and/or orbital regions most severely. The disorder is characterized by the development of marked areas of necrosis with gross softening, destruction of architecture, hemorrhage, and loss of nervous and glial elements, in association with mild inflammation. The necrotic areas may progress and coalesce, giving rise to confluent areas of cavitation. At the margins of the lesion, hypertrophied microglial cells and mild astrocytosis are seen. Perivascular cuffing with mononuclear cells is present in affected areas and characteristic type A intranuclear inclusions may be evident in oligodendroglia, less often in nerve cells. There is also a widespread mononuclear infiltration of the adjacent leptomeninges (48). Cases have been reported in which abundant intranuclear inclusions were present in cerebral tissue obtained by brain biopsy but absent at subsequent postmortem (50).

Relationship of Herpetic Infection to Other Forms of Encephalitis

The relationship of herpes simplex to acute inclusion-body encephalitis and acute necrotizing encephalitis is not clear. Although acute inclusion-body encephalitis may be etiologically related to agents other than herpes simplex, this term is frequently used as a synonym for herpetic encephalitis. The term acute necrotizing encephalitis has been used to describe two apparently different disorders; an encephalitis with severe cortical necrosis similar to that produced by herpes simplex but with no demonstrable inclusions, and an acute encephalitis which appears to begin in the white matter and is characterized primarily by involvement of white matter with marked necrosis. Cases showing lesions in both areas have been observed (46, 51). Herpes simplex has been implicated in some of the cases with cortical necrosis (46, 50). It is not clear whether acute necrotizing encephalitis represents a variety of etiologic entities or different manifestations of the same infectious process.

It has been suggested that herpes simplex virus may be related to certain forms of subacute encephalitis, namely subacute inclusion-body encephalitis of Dawson and subacute sclerosing leukoencephalitis of van Bogaert. The clinical patterns, and the electroencephalographic and laboratory features of these two diseases are essentially the same. Both affect primar-

ily children and adolescents and are characterized by an insidious onset and bizarre sensory and motor symptomatology indicative of widespread cerebral damage. Myoclonic seizures and dementia are prominent. In both the course is one of slowly progressive deterioration followed by death, usually within 6 to 12 months. The pathologic picture in these disorders also shows marked similarities. In both it consists of widespread perivascular mononuclear infiltration with neuronophagia and gliosis. The chief differences between these two diseases is the relative extent of involvement of the gray or white matter and the intensity of glial proliferation. In subacute inclusion-body encephalitis of Dawson, the gray matter is more involved and type A intranuclear inclusions are present in the nuclei of affected neurons. In the subacute sclerosing leukoencephalitis of van Bogaert the white matter is more extensively involved and type A intranuclear inclusion bodies are uncommon. Cases with features of both disorders have been observed. Current opinion is that subacute inclusion-body encephalitis and subacute sclerosing leukoencephalitis are the same disorder but that they represent different ends of the spectrum, the cerebral cortex being primarily involved in one form, the white matter in the other (48, 52). The relationship of these disorders to acute herpetic encephalitis remains in doubt. The pathologic similarities and differences between the acute and subacute forms of these diseases have been extensively reviewed by Haymaker and his associates (48).

There is evidence both for and against the concept that both forms of subacute encephalitis may represent a smoldering or chronic infection with herpes simplex virus. In favor of this concept is the occurrence of cases with pathologic features intermediate between those of acute herpetic encephalitis and subacute encephalitis (48). Additional evidence for this hypothesis is the fact that herpes simplex virus in the rabbit can induce both acute and subacute encephalitis. The fact that these disorders in the rabbit differ both clinically and histopathologically suggests that this agent may produce a similar range of manifestations in man (45). On the other hand, direct evidence implicating herpes simplex as a cause of this disorder is limited. In the few cases where viral studies have been attempted, agents have either not been isolated, or when recovered, have not been related to the illness (48, 53). Attempts to demonstrate viral antigen by fluorescent technics in brain tissue from patients with subacute encephalitis have also been unsuccessful except in one case (53, 54). In this case, both the clinical features

and pathologic findings differed somewhat from those generally associated with this disorder (53). The role of herpes simplex virus, as a cause of subacute inclusion encephalitis, therefore, still remains in doubt.

The hypothesis has been proposed that herpes simplex virus might be etiologically related to Von Economo's encephalitis (encephalitis lethargica) (55). This hypothesis is no longer held.

Localization of Herpes Simplex in Man

The development of recurrent herpetic disease in the absence of external infection indicates that herpes simplex virus remains in the tissues in occult or latent form during intervals between attacks. The tissues wherein the virus remains latent between recurrences, however, have not been clearly defined. Three possible sites have been suggested: the epidermal cells, the sensory nerve endings, and the sensory ganglia. The evidence for and against localization at these sites is summarized below.

Various attempts have been made to demonstrate that virus remains present, between attacks, in previously affected skin areas. Virus could not be demonstrated, however, by inoculating skin scrapings from sites of former lesions into rabbit corneas (56, 57). Attempts to induce lesions in man by iontophoresis at the site of previous recurrences were also unsuccessful (58). Autografting of skin from a recurrent to a nonrecurrent site, followed by parenteral administration of a pyrogen, induced a recurrence in undisturbed skin at the donor site but not in the graft at the new site, thus suggesting that virus was not present either in epidermal cells or in the sensory nerve endings (59).

Recently, Rustigian and his associates investigated this problem, using tissue culture technics (60). They failed to recover virus from skin scrapings and biopsies which were inoculated into cultures of susceptible tissues. They failed also to demonstrate virus in primary cultures of such skin after propagation for up to 5 months, as well as after exposure of these cultures to elevated temperatures ("fever"), hydrocortisone and ultraviolet light. One biopsy specimen, examined for herpes antigen by fluorescent antibody staining, was also negative. The results of the above studies suggest that virus does not persist in the skin between attacks.

The hypothesis that virus may remain localized in the sensory ganglia between recurrences appears to have strong support. Various investigators have noted that trigeminal sensory-root section for trigeminal

neuralgia is followed by facial herpes on the homolateral side in a high percentage of patients with antibodies to this agent (61, 62). These lesions usually appear on skin or mucosal areas within the sensory distribution of the second and/or third division of the sectioned fifth nerve (62). Damage to the peripheral (motor) divisions of the trigeminal nerve is not associated with appearance of herpetic lesions (62). If the second and third peripheral sensory divisions are interrupted prior to root section, the lesions also do not appear (63). Injection of alcohol into the peripheral nerve or the ganglion does not reactivate herpetic infection and, if carried out prior to rhizotomy, usually prevents reactivation (63). Finally, patients with sensory loss in the second or third division of the trigeminal nerve do not develop herpetic lesions in the affected areas following surgery (63). The above observations indicate that injuries to the posterior sensory root of the trigeminal nerve may induce herpetic lesions provided that the ganglion and the peripheral sensory divisions are intact. It is of interest that these lesions are more frequent when the posterior root section is close to the ganglion, as compared with the section near entry of the nerve into the pons (63). Although herpes simplex virus has never been recovered from the Gasserian ganglion, only a few attempts to demonstrate virus in such tissue have been made (61, 64, 65). In only one of these, moreover, was the ganglion from a patient with active herpetic manifestations tested (61). In 4 patients homolateral herpetic lesions appeared within 2 days of the time the specimens were obtained (61, 64). Of eight additional specimens tested, including six obtained post-mortem, none was obtained in association with herpetic disease (64, 65).

There is some additional data to support the concept that herpes simplex virus may localize in the Gasserian ganglion. It has been demonstrated in laboratory animals infected by the intranasal route that this agent may ascend along endoneural cells of the trigeminal nerve to the brain and that viral antigen appears in the ganglion cells when infection has progressed to the adjacent endoneural cells (40). Although a systematic study of the Gasserian ganglia of man with reference to herpetic infection has not been made, histopathologic observations on several patients have shown inflammatory changes in the homolateral Gasserian ganglion coincident with the presence of facial herpetic lesions at death (64, 66).

Several other observations suggest that herpes simplex becomes localized in sensory nerve ganglia following primary infection with this agent. The fact

that recurrent lesions in many may show a zoster-like distribution is consistent with this hypothesis (6, 67, 68). In rabbits infected by the dermal route, herpetic lesions with a zosteriform distribution may be induced by tarring the adjacent skin prior to inoculation. In such animals, virus has been demonstrated in the corresponding spinal ganglia (69). These studies suggest that virus spreads centripetally from the inoculated skin area to the sensory ganglia and then centrifugally back to the irritated skin, giving rise to the zosteriform eruption.

The observation that occasional patients develop recurring neuralgic pain in the dermatome of the affected area prior to onset of their recurrent lesions, is also consistent with posterior root involvement by this virus (57, 67, 68). This finding has been observed in 5 percent of our patients. In this regard an association has been noted in three patients between recurrent trigeminal neuralgia and the appearance of recurrent herpetic lesions in areas innervated by the affected nerves (68).

The reported occurrence and distribution of herpetic lesions in patients receiving fever therapy is consistent with a localization of this virus, either in peripheral nerves or sensory ganglia. In such patients, the lesions were characterized by their symmetry, bilateral distribution and occurrence chiefly in areas innervated by the second and third division of the trigeminal nerve (70, 71).

Several unusual cases provide further evidence for involvement of the sensory ganglia in recurrent herpetic disease. In one patient with recurrent lesions and neuralgic pain in the gluteal area, excision of the affected skin with approximation of the margins was later followed by the appearance of recurrent lesions adjacent to the former site of involvement, accompanied by the same neuralgic pain (6). In another with recurrent lesions on the dorsum of the hand, again accompanied by neuralgic pain, full thickness excision and skin graft was followed 2 years later by appearance of recurrent lesions adjacent to but not within the graft, the neuralgic pain appearing with each recurrence (6). In a third patient, with lesions on a finger and recurrences preceded by neuralgic pain in the radial nerve of the affected arm, excision of the affected area and skin graft did not prevent recurrence of the neuralgic pain, although no accompanying eruption was noted during the period of observation (56, 72). The fact that excision of the affected skin area in two of these cases was followed by a recurrence in the affected dermatome suggests that virus is not localized in skin or in sensory nerve endings between

recurrences, but rather that it is present in the sensory ganglia. The recurrence of neuralgic pain in all three patients appears to support this view.

A composite hypothesis with regard to recurrent herpetic infection as outlined by Paine is summarized below (73). During primary infection both epithelial cells and sensory nerve endings are probably involved. The virus travels centripetally in the sensory nerves along the endoneural cells, sensory ganglion nerve cells becoming infected when virus in the endoneural cells has reached the level of the ganglia. Virus remains in the ganglion cells in noninfective or occult form until activated by an appropriate stimulus. It then proceeds centrifugally down the axon of the sensory nerve to the epithelium, where it gives rise to the characteristic lesion. Data suggesting that virus may spread by means of an axonal flow (i.e., in centrifugal fashion) has recently been reviewed by Paine (73).

The fact that the mouth, perioral skin, and genital areas are the most common sites of primary infection is consistent with the high incidence of recurrences in the perioral and genital areas, the former area receiving its sensory innervation by the maxillary and mandibular roots of the trigeminal nerve, the latter from certain of the sacral roots. Primary implantation of virus into the skin through abrasion or trauma, although less common, may also be followed by recurrences at the initially affected site (6, 9, 56, 67). These findings suggest that latent virus is usually associated only with neurons innervating the site of the primary infection. The appearance of recurrent lesions adjacent to excised areas of previous involvement appears to indicate that some spread to adjacent neurons may occur. Whether virus present in a dorsal ganglion can spread to an adjoining posterior root ganglion, however, is not known.

Evidence for Latency and Recurrent Disease in the Central Nervous System of Man

The fact that herpes simplex virus may produce latent infection and recurrent encephalitis in the brain of experimentally infected rabbits suggests that it may also induce these manifestations in man (31). The appearance of facial herpetic lesions following intramedullary tractotomy and routine craniotomy in man provides support for this hypothesis; it is possible, however, that these procedures exert their effect indirectly by activating latent infection in sensory ganglia rather than in the brain (63, 74).

Several investigators have recorded a temporal association between recurrent herpetic disease and en-

cephalitic signs. Nicolau and Poincloux studied a patient during six episodes of recurrent digital herpes, each preceded by neuralgic pain in the affected limb (57). They noted that symptoms of meningoencephalitis accompanied the pain during one of these episodes. Herpetic facial lesions, occurring chiefly within the sensory distribution of the trigeminal nerve, are common during fever therapy (70, 71). Warren and his associates observed an encephalitis-like syndrome of short duration and without sequelae in patients who developed severe herpes following such therapy (70). They suggested that this encephalitis might have resulted from activation by fever of quiescent virus within the central nervous system. Janbon and his coworkers reported 2 cases of herpes simplex encephalitis characterized by apparent recurrence after 1 and 14 months, respectively (75). In a review of cases of herpetic encephalitis, Leider and his coworkers noted a possible association with reactivated latent infection in several of their patients (49). In none of the above cases, however, can the diagnosis be regarded as proven, since this diagnosis can be established with certainty only by recovery of virus from the brain or cerebrospinal fluid during episodes of recurrence.

An unusual case of recurrent psychosis of an organic type, associated with herpes simplex labialis was recorded in a 9-year-old boy by Shearer and Finch (76). His illness began with a typical herpetic stomatitis and encephalitis. Over the next 3 years, the patient had 17 recurrent herpetic lip lesions, each associated with an episode of organic psychosis. Both events occurred within 2 to 11 days of each other and the duration of the psychotic episodes (7 to 12 days) was consistent with that for recurrent herpes simplex. Clinically, the central nervous system involvement during the recurrent psychotic episodes was similar to that of the primary infection. The data in this case would appear to support the diagnosis of recurrent herpes-simplex encephalitis despite the absence of laboratory confirmation.

Two additional cases are of interest. In one, with the clinical diagnosis of herpetic stomatitis and encephalitis, mild initial central nervous system involvement was followed by recovery lasting 1 week and then by a relapse (? recurrence) (77). In the second case, which was diagnosed as subacute inclusion encephalitis, fluorescent antibody tests on postmortem brain obtained 3 months after onset of illness were consistent with the diagnosis of herpes simplex (53). This suggests that in man as in the rabbit, this virus may persist in the brain.

Herpes simplex virus has occasionally been demonstrated in the spinal fluid of patients who show neither evidence of encephalitis nor herpetic eruptions (78, 79). In the few such cases reported, data have not been adequate to determine whether the presence of virus represents subclinical central nervous system infection or whether it represents reactivation of virus at this site. Zurukzoglu recovered virus from the spinal fluid of 2 of 8 patients without clinical symptoms of encephalitis shortly after they had developed labial herpes (78). In these 2 patients it appears likely that the presence of virus represents reactivation of a latent infection in the central nervous system.

In summary, evidence indicating that herpes simplex virus may produce latent infection and recurrent disease in the central nervous system of man is at present both limited and indirect. It does, however, suggest that such infections may occur.

Related Agents Indigenous to Species Other Than Man

Agents with biologic, physical, and chemical properties similar to those of herpes simplex virus (herpesvirus hominis) have been discovered in a number of animal species and these agents are now classified together as members of a Herpesvirus Group. As with herpetic infection in man, these agents show a predilection for tissues of ectodermal origin, affecting chiefly nervous tissue, mucous membranes and, occasionally, the skin in their natural hosts. Inapparent, latent and recurrent infections with these viruses are common. With some, varying degrees of immunologic reactivity with the human virus type have been observed; others appear to be distinct. A listing of some of these viruses is given below:

Herpesvirus simiae (B virus, herpesvirus of old world monkeys, vervet herpesvirus) (2).

Herpesvirus tamarinus (herpesvirus of new world marmosets, herpes marmoset virus) (80, 81).

Herpesvirus suis (herpesvirus of pigs, pseudorabies virus) (2).

Herpesvirus of horses (equine abortion or equine rhinopneumonitis virus) (80).

Herpesvirus of cattle (infectious bovine rhinotracheitis or IBR virus, infectious pustular vulvovaginitis virus or IPV virus, IBR-IPV virus) (82).

Herpesvirus of the cat (feline rhinotracheitis virus, FRV) (83).

Herpesvirus cuniculi (virus III of rabbits) (84).

Herpesvirus of chickens (infectious laryngotracheitis virus) (85).

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Prolonged Urine Excretion of Machupo Virus by Rodents

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In the context of these discussions, particularly in the light of the reports of Dr. John Hotchin, I think a few words regarding Machupo virus might be in order.

Machupo virus is the causative agent of Bolivian hemorrhagic fever (1). It results in a fulminating illness in humans, and from 1959 through June 1964 was responsible for about 1,300 cases with 300 deaths among a population of 4,000 to 6,000 persons in north-eastern Bolivia. There is no reason to feel that it is a latent, slow, or temperate virus in humans. But some unpublished observations which we have made in our laboratory, the Middle America Research Unit, might be of interest. They were reported by Dr. Karl M. Johnson at a hemorrhagic fever symposium of the American Society of Tropical Medicine and Hygiene a month ago (2).

Although 2- to 5-day-old hamsters inoculated intraperitoneally (i.p.) or intracerebrally (i.c.) regularly succumb to Machupo virus, adult hamsters do not; in fact they show little effect. But adult hamsters inoculated i.p. with infected brain material have now been excreting 10^2 - 10^4 LD₅₀ of virus per milliliter of urine for 9 months, even in the presence of circulating virus-specific antibodies.

It has been our impression that the host-reservoir in Bolivia, again on the basis of unpublished data, is the rodent *Calomys callosus*, and that the virus is transmitted to humans from the rodent without aid from a biological vector. We have found that these rodents do not develop illness following i.c. Machupo virus inoculation. But within 15 days after i.p. inoculation virus appeared in the urine of adults and now, 150 days later, is still present. Seven of eight *Calomys* were

viremic 42 days after inoculation, and neutralizing antibodies were found on the 30th day.

Machupo virus is serologically related to Junin virus, the cause of Argentine hemorrhagic fever (3), and Tacaribe virus, an agent isolated from bats and mosquitoes in Trinidad (4). We don't know whether or not these other two agents produce a state of chronic or latent infection with viruria in rodents, but it seems that such concepts might well be extended to a wider group of human-disease-producing viruses in the future, and that the lessons learned in the study of lymphocytic choriomeningitis might be most valuable.

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DISCUSSION

KIBRICK: It is worth mentioning, in this context, that prolonged excretion of virus (viruria) has been

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demonstrated in man following intrauterine infection with rubella virus, and in infants and young children following infection with cytomegalovirus. With cytomegalovirus, the agent may continue to be excreted in the urine for as long as 3 years and such excretion may occur in the apparent absence of associated disease. During the course of an inter-

current infection with certain other agents, or under conditions that suppress the immune response, however, there may be a recrudescence of infection with cytomegalovirus. This may occur either as a sub-clinical infection or in association with obvious clinical manifestations, such as pneumonitis.

Serological, Immunological
and
Hypersensitivity Reactions in Subacute
and
Chronic Disorders of the Central
Nervous System of Animals and Man

Chairman

F. BANG

Anti-Brain Antibodies

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In spite of the fact that immunology as a science has made amazing progress in the last few years and much of its theories are solidly based on principles of protein synthesis and transfer of genetic information, it is still difficult to incorporate all of the known immunologic phenomena into one master design.

Historically, the first biological phenomenon included in the science of immunology was the development of spontaneous immunity to bacterial or viral infections. As studies of this type of immunity accumulated it became evident that specific serum proteins (antibodies) were involved in the protective mechanism developed against these harmful foreign elements. Other aspects of immunity, e.g., the tuberculin reaction, could not be explained adequately by the simple concept of an antigen/antibody reaction. Consequently, the concept of immunologically modified cells was invoked to explain the phenomenon of delayed hypersensitivity. Exactly how this phenomenon is related to serum antibodies is still a matter for debate.

Meanwhile it became evident that not all manifestations of immunity were beneficial to the individual. A "misguided" immunologic response to a foreign antigen as well as accidental sensitization to a constituent of one's own body could result in overt and often fatal disease (fig. 1).

It is a principle of immunology that a healthy body will not react immunologically against its own constituents. If for any reason "self-recognition" fails, an autoimmune reaction ensues and specific organ damage results. The formation of auto-antibodies, the reaction against "self," is thought to be the key

event in the disease "experimental allergic encephalomyelitis" (EAE). In the case of EAE it is not clear whether failure to recognize "self" or damage to the blood brain barrier precipitates the disease reaction. Experimentally, the protection which normally exists is destroyed by an injection of whole brain (or brain fraction) mixed with killed mycobacteria in a water in oil emulsion. Such an injection consistently induces extensive and often irreversible damage to the CNS. In many species it is difficult to induce disease without mycobacterial adjuvant although injections without adjuvant are capable of stimulating antibrain antibody formation.

Many animal species are susceptible to the disease, which is organ-specific (caused only by brain) but not species-specific (CNS from nonrelated species may be as effective as the CNS from the same species). The

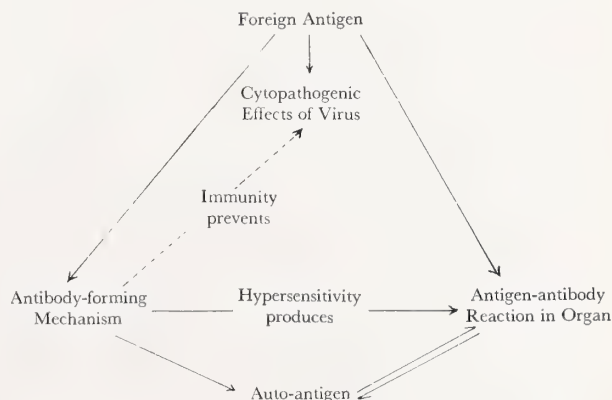


FIGURE 1.—Possible relation of foreign antigen to auto-immunity phenomena.

type of autoimmune damage observed in the CNS is an inflammatory lesion in white matter, often associated with demyelination. Many investigators consider multiple sclerosis to be the human counterpart of this condition. The latter disease is characterized by repeated attacks followed by remissions, a disease pattern which is usually not observed in experimental animals.

Even though the immunologic basis of EAE is no longer a matter for argument, only recently has it been possible to devise experiments relating disease induction to specific antigen-antibody reactions. Isolation and characterization of the encephalitogen (1) have provided us with the necessary tool for definitive studies of the problem of autoimmune pathology. Briefly, these studies can be summarized as follows:

The major encephalitogenic constituent of central nervous tissue has been isolated and characterized as a small basic protein located in the myelin sheath (2).

The encephalitogen is similar to the nucleohistones in size, charge and amino acid composition but differs from nuclear proteins in its attachment to a lipid or lipids in myelin rather than to nucleic acids. Difficulties in isolation of the free encephalitogen as a single homogeneous protein suggest that it may be susceptible to variable dissociation during the preparative procedures employed. It is obvious that size is not critical insofar as encephalitogenicity is concerned since fragments small enough to be soluble in 5 percent trichloroacetic acid yet still retaining their encephalitogenic activity have been prepared by mild enzymatic digestion (3). Neither is the attachment to lipid a prerequisite of activity. The free protein has a specific activity of the same order of magnitude as that of the protein-lipid complex (i.e., its activity per mg. protein is not altered by the presence or absence of protein-bound lipid).

Although the induction of experimental allergic encephalomyelitis is specifically dependent upon the presence of brain protein, other constituents of the injection emulsion have a marked effect on disease in certain species. In order to understand the contribution of the encephalitogen to disease induction in the guinea pig it has also been necessary to define the function of the various components of the adjuvant.

The use of Freund's adjuvant in experimental immunology is so widespread that it would be useless to attempt to document it. It is used empirically to influence the timing of antibody formation or to stimulate a specific type of antibody. It is presumed to act as a depot for slow liberation of antigen as well as to enhance cellular accumulation at the injection site.

In table I are listed the functions of the various components of the adjuvant in experimental induction of EAE.

TABLE I.—Function of Freund's Adjuvants in Disease Induction

Components	Function
Whole brain	(1) Specific antigen (2) Stabilize emulsion: high concentration—water-in-oil low concentration—oil-in-water
Water	Dissolve antigen (if water-soluble)
Paraffin oil	?—(1) Delay absorption (2) Direct absorption to lymphatics (3) Evoke mononuclear reaction
Emulsifying agent:	Stabilize emulsion: ¹
Aquaphor	water-in-oil
Arlacels	water-in-oil
Tweens	oil-in-water
Killed mycobacteria	?—(1) Induce delayed-type hypersensitivity (2) Stabilize water-in-oil emulsion

¹ Stability of emulsion is apparently more important than type. Contrary to popular belief, an oil-in-water emulsion can function effectively provided it is not too stable.

Our studies (fig. 2) have shown that other organisms can replace mycobacteria in disease induction, though not always as effectively as the latter. The amount of mycobacteria in the injection mixture is critical—excessive amounts suppress the potency of the vaccine perhaps by creating too stable an emulsion. The amount and type of emulsifier as well as the molecular weight of the hydrocarbon used are also important factors in the effectiveness of the injection mixture (4, 5, 6).

Another major goal in our study of experimentally induced autosensitization was the development of a safe sure means of suppressing the disease. Fortunately, it was discovered some time ago that intracutaneous injections of large amounts of encephalitogenic protein in saline suppressed disease to a significant degree (7).

In figure 3 are summarized schematically the suppression phenomena which are under investigation (8, 9). If one injects the animals repeatedly with purified encephalitogenic protein in saline or incomplete adjuvant prior to disease induction, EAE can be prevented (fig. 4, table II). Likewise, injections after disease induction can be shown to suppress the disease,

TABLE II.—CNS-Induced Inhibition of EAE

Specific for encephalitogenic proteins
Proportional to amount of encephalitogenic protein (inactivated by formalin) (? single or multiple protein)
Treatments before challenge better than after
Prolonged course better than short (? viscous emulsion better)
Resistant to whole body x-ray
? Passive transfer with serum

as illustrated in figure 5. Although we cannot define precisely how these suppression phenomena are related to antibody formation, it seems likely that some type of antigen-antibody reaction is involved.

Of all the immune phenomena studies, the delayed type skin reaction (similar to the tuberculin reaction in the human) is the only one which correlates well with disease onset and severity (10) (fig. 6). It can be suppressed or prevented by the same treatments which inhibit disease induction (figs. 5, 7). This observation coupled with the ability of investigators (11, 12) to transfer the disease with sensitized cells but not with immune serum indicates that autoimmune damage in EAE is related to delayed hypersensitivity rather than to circulating antibody. That is, the in-

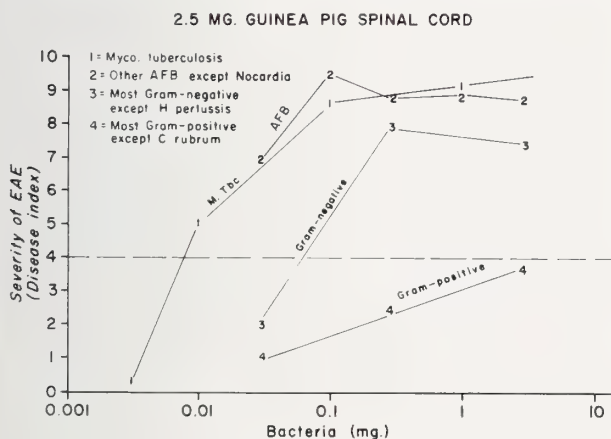


FIGURE 2.—Severity of EAE (disease index) obtained with various amounts of different micro-organisms combined with 2.5 mg (equivalent dry weight) of fresh homologous spinal cord in 0.1 ml. total emulsion.

M. Tbc: *M. tuberculosis* (mixture of strains C, DT and PN) and H₃₇Ra.

AFB: *M. butyricum*, *phlei* and *smegmatis*.

Gram-negative: *E. coli*, *S. typhosa*, *S. typhimurium* and *Pseudomonas aeruginosa*.

Gram-positive: *C. diphtheriae*, *B. cereus*, *Aspergillus fumigatus*, *Monilia albicans* and *Staph. aureus*.

(Figure reproduced from Ref. 6.)

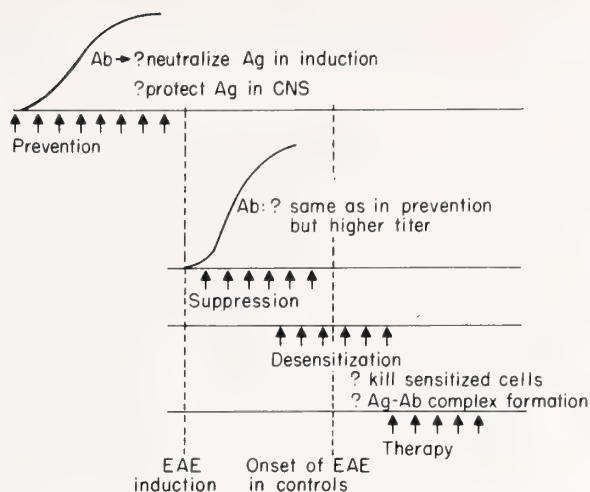


FIGURE 3.—Schematic representation of various types of inhibition of EAE and possible mechanisms. (Figure reproduced from Ref. 9.)

jection of specific brain protein with bacterial adjuvant induces primarily formation of specifically sensitized cells. If sufficient numbers of sensitized cells gain access to the CNS, marked inflammation occurs with resulting damage to the tissue. Serum antibody may be formed as a coincident reaction but it appears to have little significance so far as disease induction is concerned. Its formation, however, may actually be of great importance in protective mechanisms (13).

The main thread of continuity which has been maintained through all of these studies has been the search for the antibody or antibodies which induce disease as well as those which suppress or inhibit the reaction. We have not been alone in this search. Many antigens have been used in attempts to study the antibodies in EAE (table III).

TABLE III.—Antigens Used in Antigen-Antibody Reactions in EAE

Encephalitogenic	Non-encephalitogenic
Whole CNS	Alcohol-soluble lipid hapten, cerebroside
Myelin	
Phospholipid-protein complex	Saline extracts of CNS
Basic myelin protein	Trypsin digests of CNS
	Proteolipid protein
	Trypsin-resistant protein residue (TRPR)
	Neurokeratin
	Ganglioside

Inhibition of EAE by Pretreatment with Whole CNS or Acid Extract (Challenge: 2.5 cord, 1.0 Tbc)

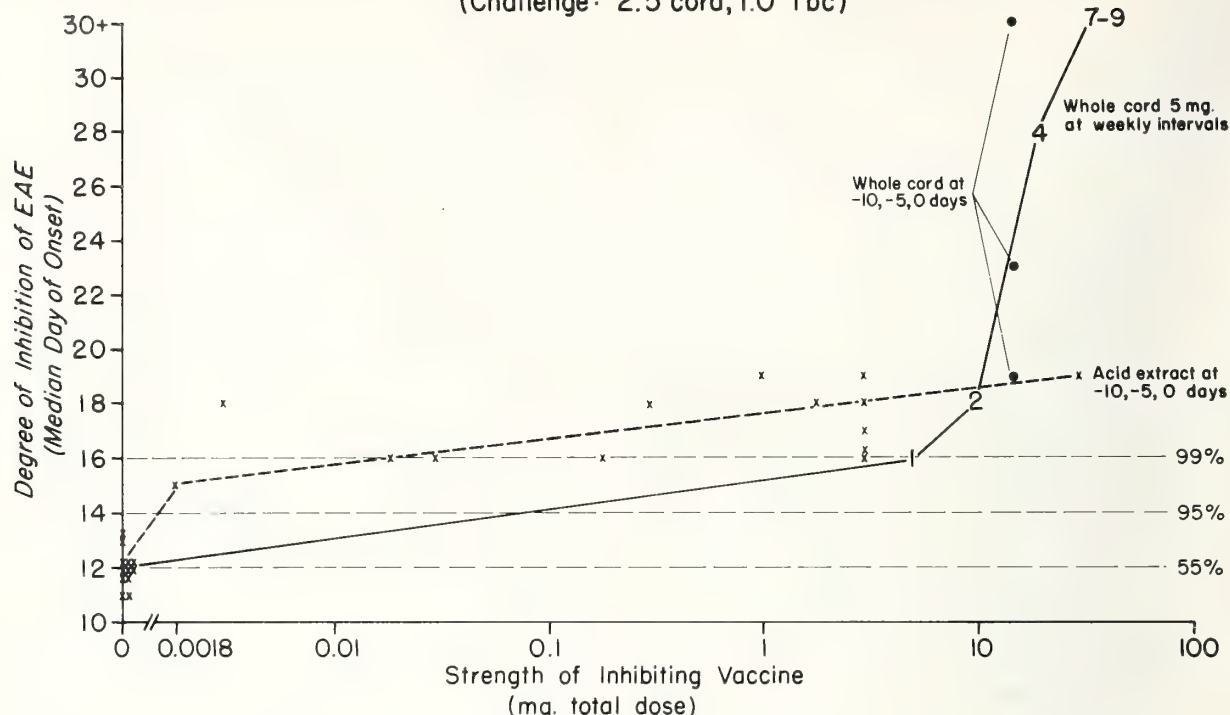


FIGURE 4.—Inhibition of EAE by pretreatments with W/O emulsions of varying amounts of whole CNS or acid extract (1). Several experiments are summarized. The median day of onset of EAE in untreated controls was 12 days in 55 percent of experiments, 14 days in 95 percent and 16 days in 99 percent of experiments (horizontal dashed lines). With acid extract given at -10, -5, and 0 days before challenge, significant delay in onset of EAE was obtained with as little as 0.0018 mg., but even with 30 mg. (7) not so much delay as could be obtained in 3 experiments with 2.5 mg. of whole CNS over the same time schedule. Whole CNS given at weekly intervals for 1, 2, 4, 7 or 9 weeks before challenge produced increasing inhibition of EAE. (Figure reproduced from Ref. 8.)

Since some of these are nonencephalitogenic, it is little wonder that in many of the experiments no correlation existed between antibody and disease. In table IV are listed all of those antigen/antibody reactions which have been reported in EAE. Many of these are now being studied with purified encephalitogen in several laboratories. Basic proteins similar to the one we have isolated from guinea pig CNS have also been isolated from bovine (24), rabbit (15) and human sources (25). Thus it is possible to compare the behavior of heterologous as well as homologous systems and to study cross reactivity among these preparations.

Antibrain antibodies have been reported in many human diseases as well as in EAE (table V). Whether or not actual damage to the CNS can be ascribed to the presence of circulating antibody in these conditions is beside the point—the important fact is that, if anti-brain antibody is present, an autoimmune mechanism has been invoked and is operating. Such a mechanism might be involved in the delayed onset of CNS damage

TABLE IV.—Antigen-Antibody Reactions Reported in EAE

Circulating Antibody	Cellular Hypersensitivity
Complement-fixation (14)	Delayed-type skin reactions (10)
I ¹³¹ Ag-Ab complex pptn. (15)	Passive transfer of cells (11, 12, 20)
Gel-diffusion, precipitation (9, 16)	Contactual agglutination in tissue culture (27)
Hemagglutination (9)	Demyelination by cells in tissue culture in EAN (22)
Passive cutaneous anaphylaxis (17)	Antigen uptake by cells in tissue culture (23)
Fluorescent globulin attachment to myelin (18)	
Demyelination by serum in tissue culture (19)	
Inhibition of EAE by serum (13)	

following slow viral infections. The encephalitogenic protein may not be the antigen in these cases. There are many proteins unique to the CNS which could,

Desensitization of skin reaction and EAE

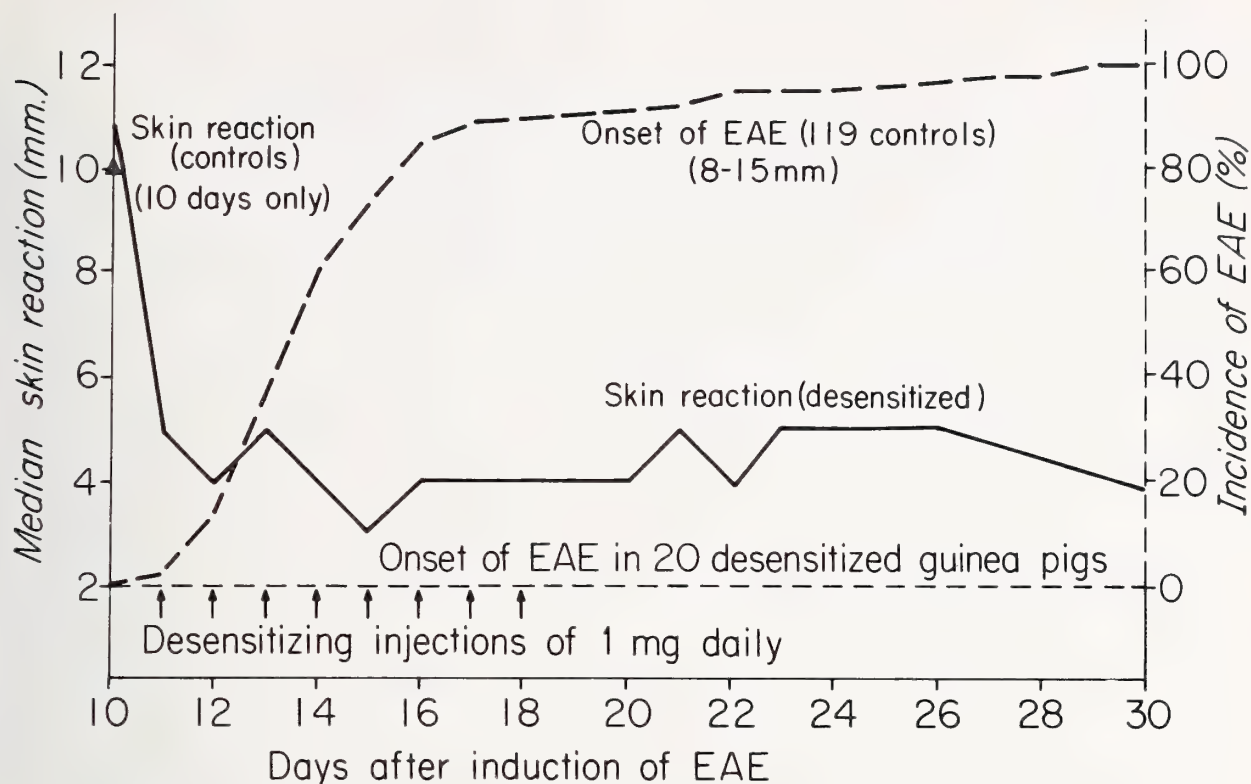


FIGURE 5.—Effect of daily injections of homologous basic protein (#260, 1.0 mg./day) at 11 to 18 days on skin reactivity to the same antigen (0.015 mg./0.1 ml.) and on the onset of EAE induced with the same protein in complete Freund's adjuvants. (Figure reproduced from Ref. 10.)

TABLE V.—Incidence of Anti-Brain Antibodies Reported in Human Diseases

Antibodies	Disease	Incidence in disease	Incidence in controls
Anti whole brain (26, 27)	Multiple sclerosis	14/59	0/34–2/60
	Guillain-Barré	19/38	
	Systemic lupus erythematosus	9/12	4/183–23/608
	Cirrhosis	7/18	
Anti brain lipids (28, 29, 30)	MS, encephalitis & polyneuritis	42/104	1/82–7/260
	Multiple sclerosis	1/69	0/50–4/50
	Multiple sclerosis	12/84	?
Demyelinating (19)	MS—active	25/37	
	MS—? active	12/30	1/27–2/28
	MS—inactive	0/33	9/44
	Amyotrophic lateral sclerosis	9/15	
Antigangliosides (31)	Multiple sclerosis	8/42	0/42
Antiasialogangliosides (31)	Viral encephalitis	8/16	
	Tay-Sachs	5/14	0/42
Anti gray matter (32)	Carcinomatous sensory neuropathy	4/4	1/30

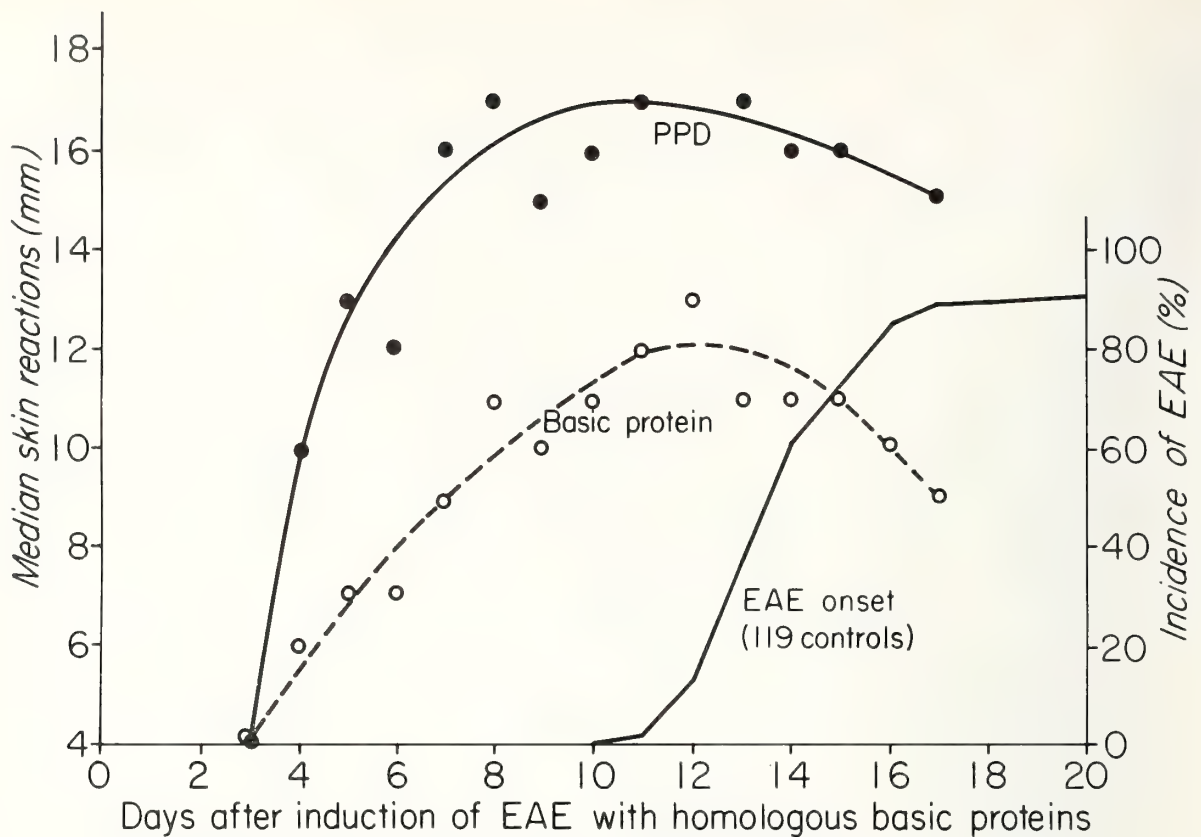


FIGURE 6.—Onset of skin reactivity to homologous basic protein and PPD and of EAE following induction of EAE with homologous protein in complete Freund's adjuvants. (Figure reproduced from Ref. 10.)

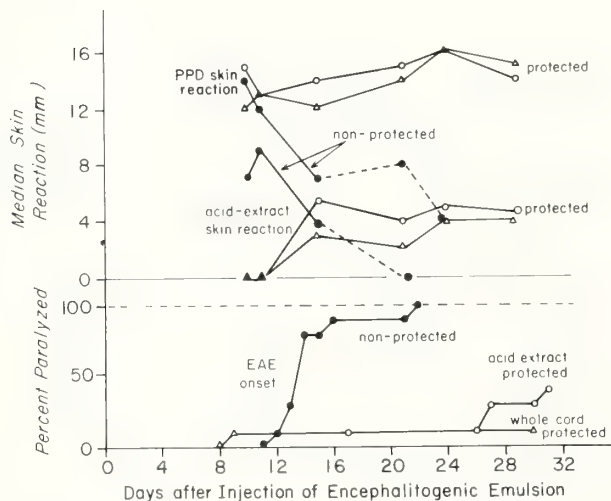


FIGURE 7.—EAE onset and skin reaction to tuberculin and heterologous acid-extract in protected and nonprotected guinea pigs. (Protection induced by twice weekly injections of heterologous acid-extract or homologous spinal cord for 3 weeks before and after induction of EAE by homologous spinal cord with Freund's adjuvants.)

as a result of viral invasion, become involved in an autoimmune reaction. It is conceivable that the characteristics of the pathological lesion might vary with the protein involved, thus explaining the multiplicity of lesions observed. Even if the basic encephalitogenic protein were not involved the methods and results of the present investigation might well be useful in studying the etiology of CNS damage in the slow viral infections.

SUMMARY

The present state of knowledge regarding the participation of antibodies in induction of the experimental disease, allergic encephalomyelitis (EAE), has been reviewed briefly. Earlier attempts to correlate anti-brain antibodies with the course of disease were unsuccessful because crude antigenic mixtures or, in many instances, nonencephalitogenic brain fractions were used to induce antibody formation.

A purified encephalitogenic protein isolated in our laboratory has been used in a study of delayed hypersensitivity related to disease onset and various types of suppression of EAE. The antigenicity of this protein

is currently being studied by other techniques designed to detect various types of serum antibody.

EAE can be suppressed in experimental animals by injecting the purified protein without mycobacteria prior to the induction injection (which contains mycobacteria and active brain fraction). Suppression or desensitization can also be achieved by other injection time schedules.

If an autoimmune phenomenon were responsible for brain damage in the latent or slow viral infections, the biochemical and immunochemical techniques used in studying EAE might help in the solution of this problem.

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DISCUSSION

Q-1: You mentioned antigen-antibody complexes. Are these demonstrable in the area of the lesion?

KIES: I do not believe this has been demonstrated yet. I cited the work of Dr. Kibler (15) who has used I¹³¹ labelled antigen to demonstrate antibody in the serum of rabbits. But this was not a diagnostic test, merely a study of the development of serum antibody during the interval of disease induction.

Q-2: What animals did you use? Can you induce EAE in mice?

KIES: We have studied guinea pigs almost entirely. Occasionally rats and rabbits are used in our experiments. Mice can be used but have not been considered as satisfactory as guinea pigs. Olitsky, Lee (33) and Schneider (34) at Rockefeller Institute have studied the disease in inbred strains of mice. Other people have had difficulty in maintaining a susceptible strain in their laboratories.

Q-2: No skin test in mice?

KIES: So far as I know, the skin test has not been successfully demonstrated in mice.

BANG: One of our tasks in this group is to try to bring together the ideas of virology and genetics with the immunological aspects of disease. In the light of that I wonder if you might tell us a little more about the genetic story.

KIES: I do not feel that I can discuss the genetics of EAE adequately. The Hartley strain guinea pig which is an albino strain but not an inbred line is susceptible. NIH mixed color guinea pigs which are randomly bred in a closed colony have been uniformly susceptible to EAE in our experiments. There are two inbred strains of guinea pigs begun in 1906 by Sewall Wright, a biologist in the Department of Agriculture, and maintained in a more or less inbred condition over the years until the mid-thirties when they were taken over by NCI. In 1956 the breeding of these animals was taken over by the NIH Animal Production Section at Dr. Freund's instigation, and they are now being maintained here at NIH as Strain 2 and Strain 13. Strain 2 is almost completely resistant to EAE, whereas Strain 13 is about as susceptible as Hartley's and NIH mixed color guinea pigs. Dr. Sanford Stone in NIAID is using these guinea pigs in genetic studies on hypersensitivity and antibody formation. The genetic picture in mice has been studied by Drs. Lee and Schneider at Rockefeller Institute (33, 34).

BANG: The reason I brought up this point is that I know Dr. Dubos has established the fact that different strains of mice may differ in their susceptibility simply on the basis of their original bacterial components. So it is possible that different strains of animals can differ by mechanisms other than the true Mendelian genetics and I was interested in the extent of the knowledge on this point.

KIES: Dr. Lee has studied certain differences in the BSVS and BRVR mice which may be pertinent to this problem (35).

PATERSON: I think a point to make is that the mouse is a very difficult animal to start out with if one wants to work with this disease. Out of all the various strains available, the BSVS strain of Lee, Schneider and Olitsky at the Rockefeller Institute goes better than the rest but still it has to be primed like a pump. It has to receive several injections and it has to be hit with pertussis vaccine. Even then it develops a disease with meager histopathological responses and a clinical pattern which is very

mild. I think, in response to Dr. Bang's comment, that one thing that has impressed us about working with mice strains, and we have worked with eight or ten over the years, is that we can take a given one, let's say the C-57 black inbred strain of mouse, and bring it up to the BSVS level if we do anything that tends to cause a marked proliferative response in the lymphoid tissues. This is exactly what your

latent microbial agent could be doing silently, and certain strains seem to be more susceptible than others. I think that what priming of the BSVS strain with pertussis vaccine is doing, in essence, is causing an intense proliferation of lymphoid tissues leading to increased numbers of those few cells that are responding to brain and capable of making the critical immune response.



Scrapie and Allergic Encephalomyelitis¹

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Is there any conceivable relationship between scrapie and experimental allergic encephalomyelitis? On the surface there would seem to be little basis for raising the question. Scrapie is believed to be a neurotropic virus disease spontaneously arising in sheep (1) or experimentally passaged to goats and mice by means of scrapie-infected brain suspension (2-4). Allergic encephalomyelitis (AE) is widely accepted as an immunological disorder experimentally induced in rats and other mammalian hosts by often repeated injections of normal brain or a single injection of brain emulsified in Freund's immunologic adjuvants (5-7). The precise mechanism, however, underlying each condition and the nature of the inciting agent—be it microbial parasite or tissue antigen—remain to be identified with certainty. These facts together with the unusual biologic features associated with both scrapie and AE make it worthwhile to step outside the limits of prevailing opinions in hopes of gaining additional insight into the etiology of each disease.

The remarkable heat stability of scrapie agent and existence of incubation periods ranging in months or years are unusual properties for a viral agent and sug-

gest that factors other than those associated with classical infection might be implicated. Certain properties reported for the scrapie virus are reminiscent of those reported for the encephalitogenic antigen responsible for AE and suggest that the evidence upon which the infectious theory of AE was excluded years ago be reexamined. Finally, the development of immunological tolerance as an additional biological dimension and its vital role in the interplay of viral agent and respective immune response, so well described by Traub (8), Hotchin (9) and Volkert (10) for lymphocytic choriomeningitis virus infection of the mouse, lead one to ask whether these concepts have potential application to scrapie and AE. During the incubation period of many months, does the scrapie-infected but as yet still asymptomatic host lose preexisting immunological tolerance to the virus? Are immune responses made not only to the virus but nervous tissue in which the virus resides? Could the occurrence of central nervous system disease be the result of an interaction of immune responses made by the sensitized host with the virus or a viral-nervous tissue complex? Could such events, including participation of the scrapie virus or a similar type of agent, conceivably occur in the development of AE?

This presentation will briefly: (a) restate prevailing opinion regarding pathogenesis of AE, (b) review some of the evidence that AE lacks an infectious basis, (c) draw attention to certain similarities between scrapie virus and the encephalitogenic agent responsible for AE and, finally, (d) summarize data from our laboratories and current thoughts concerning possible relationships between the two diseases.

Current Opinions About AE.—Disseminated encephalomyelitis, the equivalent of AE, may be induced

¹ Experimental studies done by author supported in part by U.S. Public Health Service Grant B-3104 of the National Institute of Neurological Diseases and Blindness, National Institutes of Health. No attempt will be made in this paper "to cover" the voluminous literature dealing with scrapie and allergic encephalomyelitis. References will be cited which provide a great deal of information about the general topics or specific issues under discussion. ² Each of these references will provide the especially interested reader with an ample number of additional readings.

² Studies by author in mice were carried out during tenure as Career Scientist of the Health Research Council of the City of New York, New York University School of Medicine, New York, N.Y.

in laboratory animals by often repeated injections of nervous tissue (11, 12). Since introduction of adjuvants in 1947, the disease is routinely induced in many mammalian hosts by a single injection of nervous tissue in company with Freund's adjuvant consisting of killed mycobacteria in paraffin oil (see reviews (5-7)). Many of the animals exhibit paralysis. Most can be shown to produce antibrain antibodies. Virtually all of the sensitized animals develop characteristic microscopic lesions of AE consisting of perivascular round cell infiltrates, associated parenchymal microglial reactions and varying degrees of perivascular demyelination.

The evidence is overwhelmingly great that AE is the result of an immunological response made by immunopotent lymphoid tissues against unique antigenic constituents in the nervous tissue-adjuvant inoculum. The encephalitogenic antigen is organ-specific but not species-specific. That is, it is largely restricted to nervous tissue but found in the brain and spinal cord of most mammals. The seat of the immune response appears to be the lymph nodes since AE may be transferred in its entirety from afflicted to normal animals by means of lymphoid cells from appropriately sensitized donors (13). The actively induced form of the disease can be viewed as an immunological "attack" by lymphoid tissue of the injected host against his own neuraxis. From a biological standpoint, the host is led inadvertently to reject his own brain because it contains the very same antigen as that in the brain inoculum which led the host to make the immune response in question. An unspecified number of lymphoid cells—the cells themselves or antibodies produced by them—are believed to penetrate the blood-brain-barrier and interact with encephalitogenic antigen. The critical event is the interaction of living cells or free antibody with specific antigen in the nervous system (14). The result is injury and consequent inflammation with development of the identifying lesions of AE. The encephalitogenic antigen is widely accepted as being a native constituent of nervous tissue serving to endow special antigenic activity upon this organ system. Indeed, recent evidence has convincingly localized the antigen to myelin or the glial cells which are implicated in myelin synthesis and maintenance (15, 16).

Evidence That AE Lacks an Infectious Basis.—On one side of the coin can be found a large body of evidence against any infectious agent being implicated in AE. Some of the more pertinent observations are as follows (5-7). There is no evidence for spontaneous transmissibility of the condition or experimental "pas-

sage" of AE with nervous tissue of afflicted animals. The organ-specific character of the disease unaccompanied by species-specific restrictions as to host susceptibility or major identifying morphologic features points away from an infectious process. And the extraordinary resistance of the encephalitogenic factor to conditions inimical for living matter, such as exposure to organic solvents or formaldehyde over a wide pH range and prolonged autolysis or boiling argues against an infectious agent being involved. The finding that brain extracts held at 100° C. retain encephalitogenic activity especially seems inconsistent with a microbe having an etiological role.

It should be recalled that from a historical standpoint the discovery of acute disseminated encephalomyelitis, the forerunner of AE as we know it today, resulted from attempts to determine how certain viruses (such as variola and vaccinia) occasionally resulted in encephalomyelitic neurologic complications in man (11, 12). Evidence over the years has more and more indicated that the histological changes characteristic of AE experimentally produced by nervous tissue sensitization of animals is closely similar, if not identical in some instances, to the acute encephalitis closely associated in time with smallpox, smallpox vaccination, measles and chickenpox infections of man (17). Thus, the laboratory animal model under discussion is deeply interwoven with viral infection.

Most of the evidence against an infectious component to AE was collected before current and advanced methodology was available for detection of microbial agents. The evidence should be reevaluated in the light of information now rolling in on scrapie and other "slow neurotropic viral agents" which has served to amplify preexistent views concerning the biological properties of viruses. This is clearly brought home by data showing stability of scrapie and analogous viruses to heating at 100° C. Another unusual feature of scrapie is the characteristic long incubation period, ranging up to many months or years. Of special interest in this regard is the fact that mice and guinea pigs inoculated parenterally and/or intracerebrally with brain tissue from animals with acute disseminated encephalomyelitis in hopes of demonstrating a transmissible agent were observed usually for periods of only 6 or fewer months (11, 12), that is, less than the minimum incubation period of scrapie.

Similarities Between Scrapie and AE.—Some properties of the agents responsible for scrapie and AE are shown in table I. They have been selected with an eye not only as to their similarities but because they are not easily reconciled with either a strictly infectious

TABLE I.—Some Properties of Scrapie Virus and Encephalitogenic Antigen

Properties associated with agent responsible for each disease	Scrapie	Allergic encephalo-myelitis
Agent reported present in:		
Brain	++++	++++
Spinal cord	++++	++++
Lymph nodes	+	+
Liver	+	+
Skin	?	+
Kidney	0	+
Testis	0	+
Minimum latent period (without use of adjuvant)	7 months	5 to 7 months
Resistant to boiling	Yes	Yes
Induces circulating antibodies (detected by routine tests)	No (Mice)	No (Lewis rats and guinea pigs)
Neutralized by convalescent serum	No	No

etiology for scrapie or an immunological etiology for AE. Based on infectivity titrations of tissues from experimentally infected mice reported by Eklund et al. (18), the factor responsible for scrapie is present in high concentration within the brain and spinal cord. Scrapie agent can also be found in other tissues, e.g., lymph nodes and liver, the amount of virus running two or three logs less than that within the neuraxis. Virus appears to reside less often in kidney or testicular tissues.

In the case of AE, the responsible factor or antigen is usually considered to be restricted to nervous tissue. Evidence of sharing of antigenic activity between brain and other tissues, however, can be found in the immunologic literature and deserves emphasis in this discussion (table I). Lewis (19) showed that brain and testicular tissue extracts contain a common antigen. When injected into rabbits, these tissues called forth antibodies which fixed complement with extracts of either brain or testes *in vitro*. Work in our laboratory has confirmed this finding and shown that such cross-reactivity can exist *in vivo* (20). For example, of 33 rats sensitized intracutaneously to guinea pig testis emulsified in complete adjuvant and examined histologically 21 or 22 days later, 2 were found to have focal vascular lesions in the brain indistinguishable from those of AE induced in the usual fashion. None of the 33 rats developed aspermatogenesis, confirming the observation of prior workers that induction of this disease requires the use of homologous testes plus

adjuvant. It should be stressed that induction of AE following transfer of lymph node cells from donors sensitized to nervous tissue-adjuvant is probably due to immunological responses elaborated by the cells. This point, however, remains to be established and does not alter the fact that injection of appropriately sensitized lymphoid cells leads to AE. Noteworthy are other reports describing the occurrence of AE in guinea pigs following sensitization to adjuvant alone or adjuvant combined with skin or kidney (21-23). While there is a great temptation to deemphasize the significance of such observations because they do not fit prevailing thoughts about the organ-specificity of encephalitogenic antigen, they must be considered in the sorting of all threads of evidence touching on the pathogenesis of AE.

A minimum latent period of around 7 months usually elapses after introducing scrapie virus into a susceptible host before clinical evidence of the disease appears. Before the use of adjuvant, from 5 to 7 months were required after initial sensitization to nervous tissue before the appearance of clinical signs of acute disseminated encephalomyelitis in monkeys (11, 12). Although the eventual occurrence of paralysis in these animals is still thought to have resulted from repeated injections of nervous tissue (which served to heighten nervous tissue sensitization to a critical level required for the production of disease), there is no reason to exclude the possibility that a period of 5 to 7 months was required before the effects of the initial injection of brain could be manifested.

As already mentioned, both the scrapie agent and the encephalitogenic antigen are extremely stable and retain their respective activities after heating at 100° C. or higher.

It is of considerable interest that so far no demonstrable immune response directed against scrapie virus has been reported in experimentally infected mice or other susceptible hosts (24). Sera from mice with clinical signs of scrapie infection do not fix complement specifically with extracts of scrapie-infected brain used as antigen. It should also be emphasized that some hosts, e.g., Lewis strain rats and Hartley guinea pigs, which are noted for their susceptibility to AE, do not produce immune responses against encephalitogenic antigen that are demonstrable using conventional complement-fixation or precipitating-type systems for immune sera assays (25).

Finally, neither the agent of scrapie nor that responsible for AE can be "neutralized" by exposure *in vitro* to sera from animals stricken with AE or scrapie. The

absence of a circulating antibody against scrapie together with lack of neutralizing activity of sera from afflicted animals constitutes a conspicuous gap in the chain of evidence that scrapie is a virus disease. Sera from some strains of animals with AE, e.g., the CFN strain rat, regularly do contain complement-fixing antibody against brain in high titer (26). Such sera have the capacity to prevent or suppress development of AE when injected every other day in relatively large amounts into other sensitized rats (26). This method of demonstrating a protective effect of serum has not been applied to the scrapie problem and efforts to do so might bring rewarding results.

Do Scrapie Mice Develop Immune Responses to Nervous Tissue?—An opportunity to get a preliminary answer to this question was provided by Dr. Joseph E. Smadel and Dr. J. A. Morris of the Division of Biologics Standards, National Institutes of Health, in 1962. As shown in table II, eight mice representing three different strains were studied some 11 months following intracerebral inoculation of scrapie-infected

TABLE II.—Absence of Antibrain Antibodies and Lesions of Allergic Encephalomyelitis (AE) in Scrapie Mice

Mouse No. ¹	Mouse strain	Clinical status	Units of complement fixed with brain antigen ²	Occurrence of microscopical lesions of AE ³
1	CD	Ill; neurological signs	0	none
2	C57B1	Ill; neurological signs	0	none
3	CD	Ill; neurological signs	2	none
4	GP	Ill; neurological signs	0	none
5	C57B1	Clinically well	0	none
6	C57B1	Clinically well	0	none
7	C57B1	Clinically well	4	none
8	C57B1	Clinically well	2	none

¹ Inoculated 8–15–61 with scrapie-infected brain suspension; onset of neurological signs early June 1962. Bled and sacrificed 6–28–62.

² Number of 50 percent hemolytic units of complement (C'H₅₀) fixed, of exactly 100 C H₅₀ available, by 0.1 to 0.35 ml. of mouse serum in presence of ethanolic extract of normal mouse brain used as antigen.

³ Multiple H and E stained sections from 3 blocks of brain and 4 blocks of spinal cord examined microscopically for focal vasculitis and associated microglial reaction characteristic of AE.

brain tissue. Numbers 1 through 4 were just beginning to exhibit florid clinical neurological signs of scrapie whereas the remaining mice were clinically well when all were bled and sacrificed. None of the sera were found to have complement-fixing antibodies reactive with mouse brain used as antigen. In the assay system used, specific fixation of 10 or more hemolytic units denotes significant levels of antibody. Multiple sections of brain and spinal cord from each of the eight mice revealed no histological changes suggesting AE. Two of the four mice with neurological signs, however, did show astrocytic and other changes consistent with early scrapie.

Based on these data, scrapie mice do not develop immune responses and/or lesions of the type anticipated in mice sensitized with nervous tissue plus adjuvant. The absence of adjuvant in the original inoculum, of course, could be a major factor accounting for these negative results.

Could Scrapie and AE Represent an Immune Response to a Common Latent Neurotropic Agent?—As shown schematically in figure 1, for the purposes of discussion, let it be assumed that scrapie virus resides in virtually all mammals and is passed from latently infected mother to offspring. The host would be immunologically tolerant of the agent and show few if any clinical signs of illness. As a preferential neurotrophe, the virus lives on in the neuraxis without eliciting immunological response—a situation analogous to that of lymphocytic choriomeningitis virus (9).

Injection of brain containing latent scrapie virus if emulsified with Freund's immunological adjuvants, might well result in loss of the scrapie-tolerant state due to the action of the adjuvant. Adjuvant has been shown in several situations to abolish or materially weaken tolerance to various antigens such as pneumococcal polysaccharide and synthetic polyamino-esters (27, 28). Loss of tolerance to the scrapie agent would lead to a vigorous immune response to viral antigen in the brain inoculum. Interaction of the immune response with scrapie virus in the brain of the sensitized host would lead to injury and an acute encephalomyelitis recognized as hyper-acute scrapie or AE. Injection of brain tissue alone (without classical Freund-type adjuvants) might well have an adjuvant-like effect because of the various lipids and proteolipids contained in brain. A gradual loss of tolerance to scrapie would occur with a less vigorous immune response against the virus leading to a more chronic type of neurological disease, viz., classical scrapie.

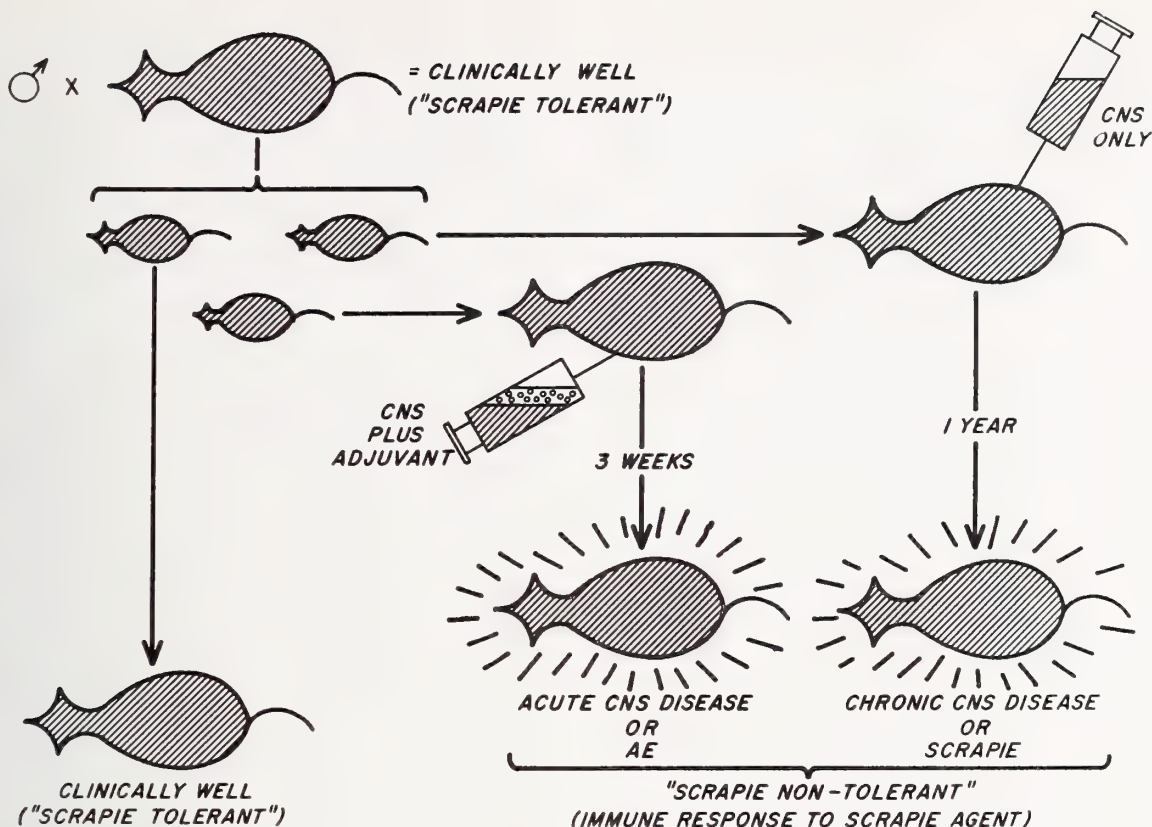


FIGURE 1.—Schematic presentation of scrapie and allergic encephalomyelitis (AE) as immune diseases involving loss of tolerance to a common latent neurotropic agent.

SUMMARY STATEMENT

The thoughts expressed in this paper have been deliberately drawn to provoke discussion. I am aware that the "half-life" of any theory is inversely related to its validity and that the real value of theoretical discussions lies in forcing a reexamination of old facts and observations in attempts to see new relationships and meanings. Will isolated scrapie virus combined with adjuvant and injected into a suitable host lead to a disease resembling scrapie or AE? Does scrapie-immune serum when added to myelinating brain cultures (of the type described by Bornstein (16)) cause demyelination resembling that produced by AE-serum? Is it possible that serum from animals with AE and which have made a vigorous immune response to nervous tissue constituents (including antigenic determinants of a latent virus) "neutralize" scrapie virus and prevent its transmission to a susceptible host?

Answers to these questions may not necessarily reveal a close relationship between scrapie and AE. They will surely provide more information essential for further understanding of each disease.

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DISCUSSION

BANG: Might I ask you whether or not you would exclude the idea that the virus might stimulate the allergic process simply by having an antigen in common with that of nervous tissue?

PATERSON: That is a definite possibility. Although no examples can be cited at present, we should be aware of the well-established cross reactions between certain streptococci and heart muscle or kidney tissue.

HOTCHIN: I was very interested to hear Dr. Paterson's remarks. We have thought along these lines for some time with respect to LCM and have tried to pin the system down. We have thought about the possibility that the virus induces a changed cell surface similar to that induced by influenza, or by polyoma virus. We have looked for this type of event, without success. On the adjuvant question, we have injected adjuvant at different times during the life of the LCM-tolerant animal in an effort to accelerate the late onset disease that we see. For a while the animals did get sick; they looked as though they had the late form of disease and had an anemia which appeared to be hemolytic. However, the mice started to get better again. Whatever effect we observed was transient, lasting only 2 or 3 weeks and then passing off.

BROTHERSTON: It is a very attractive theory. We do know that animals with chronic diseases, e.g., tuberculosis, can produce auto-antibodies reactive with the organ and tissues which are affected. We also know, of course, that the presence of auto-antibodies in the animal does not mean that the particular organs need be affected. One point I would like to make is that there are very marked differences between allergic encephalomyelitis and the scrapie syndrome in sheep. For example, we have attempted to induce scrapie by the transfer of lymphoid cells from affected animals to other animals and

have failed to do so. Another point is that a great many animals in our country are inoculated with vaccines of one type or another, combined with adjuvant. This is true both in the field and experimentally. Very few animals die in our Institute that do not get their brains examined, and we have not seen anything that would lead us to believe that we could produce evidence for this type of theory.

ZLOTNIK: I would like to quote two experiments which I think are crucial. In one experiment, a number of sheep were divided into two groups. One group got normal sheep brain with Freund's adjuvant; the other group got scrapie brain and adjuvant. After 3 weeks, we had cases of allergic encephalitis in both groups. One or two of them died and the remainder recovered. Following that, 6 months later, we had 30 percent of cases of scrapie in the scrapie-brain group in animals that had recovered from EAE. The second experiment we did in another way. We took animals that had been injected subcutaneously with scrapie brain, and animals injected with normal brain. About 4 months after the inoculation of scrapie, we challenged both groups with normal brain plus adjuvant. We observed cases of scrapie and allergic encephalitis concurrently in one group and just allergic encephalitis in the other. Therefore, I would conclude that there is no connection at all between allergic encephalitis and scrapie-like diseases.

PATTISON: I have had the extremely embarrassing experience in experiments with scrapie over the years of using formal control animals inoculated with normal brain and, on five different occasions, using

four different brains, I have had scrapie develop in the control animals. I have no reason to believe that there could be any contact and that the brains were other than normal. I find now that Dr. Paterson has a possible explanation for this, and this is a most astonishing development. I do think it one that must be borne in mind and I, personally, cannot overlook it. Although we are, I think, talking about the scrapie virus, perhaps it has yet to be shown that the virus is the whole story.

PATERSON: The failure to transmit any condition with lymph node cells within something other than isohistogenic strains of animals does not carry a lot of weight. Many years went by trying to transfer allergic encephalomyelitis until the prerequisite of histocompatibility between donor and recipient was recognized. I think Dr. Zlotnik's report is strong evidence against the concepts I have advanced. It should be mentioned, however, that AE can have a relapsing type of course. For example, we have seen acute AE in an animal such as the cat, followed by rapid recovery, and then, at the time you would be seeing your scrapie, we have observed a slower type of neurological disease appear. We are forced to call it AE, in terms of what has gone before. But, when we sacrifice the animal, we do not see changes of acute AE. Rather, astrocytic proliferation and demyelination begin to be conspicuous findings.

ZLOTNIK: The thing that I wanted to stress was that experimental allergic encephalitis, as we know it, does not seem to have a very direct connection with scrapie because we can produce it independently in the same animal at two different periods.

Observations on Mouse Nerve Tissues in Culture ¹

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When Dr. Gajdusek invited me to participate with you in this symposium, I answered that we had not yet applied our particular method to the study of viruses and their possible effects on cultured mammalian nervous tissues. However, preparations for these experiments had been in progress for about 4 years with observations on tissues obtained from the mouse which, as you know, is particularly susceptible to a number of virus agents. Today, I will try to present to you examples of the kinds of model systems available for exploitation by various disciplines which should certainly include virology.

The method of maintaining explanted fragments of nerve tissues in the Maximow slide assembly (1, 2, 3) is demanding, but rewards the investigator by (1) favoring differentiation and maturation; (2) exposing the fragment to easy manipulation of its environment; (3) constantly offering the living events for observation and photographic recording at the cellular level during the months of the tissues' *in vitro* existence, and, as a corollary to the last, by permitting a selection of any predetermined stage of the active or reactive events for examinations by other techniques such as electron microscopy (4, 5, 6), microelectrophysiology (7, 8, 9), or microchemistry (10, 11).

Comparing fragments from different levels of the neuraxis reveals that each has an optimal moment for successful explantation. The excessively young are too susceptible to the shock. Many cells succumb and the decrease in cell population may compromise the fragment's future *in vitro* development. Conversely, tissues that are too old tend to remain thick.

The inadequate diffusion of metabolites and oxygen from the liquid environment produces areas of central necrosis. Unfortunately, no way has been found to foretell the best age for the various levels of the neuraxis. Empirically, the 12- to 13-day-old embryo myotomes for cord-muscle preparations, the 16-day-old embryo for spinal cord alone, the newborn cerebellum and the 2- to 3-day-old cerebral neocortex appear to produce the best results.

In brief, each type of tissue is explanted into the Maximow slide assembly, incubated in the lying-drop position at 34° to 35° C., removed to room temperature each day for microscopic observations and evaluation, and fed twice a week with a fresh drop of nutrient medium composed of 35 percent Simm's balanced salt solutions (pH 6.9-7.1), 25 percent bovine serum ultrafiltrate; 40 percent serum (human placental, fetal calf, horse), and a glucose supplement to yield a final concentration of 600 mgm. percent. No antibiotics are used.

The primary question asked of the cultured materials is the extent and degree of faithfulness with which they reproduce the known characteristics of the tissue as observed *in vivo*. Many studies (3, 12, 13, 14, 15, 16, 17, 18, 19) have presented the *in vitro* characteristics of the individual cellular elements—neurons, glia, Schwann cells, microglia, ependyma—as well as the establishment of significant intercellular relationships within the community of this mixed population. In many details, the patterns of cytological, ultrastructural and bioelectrical development remain faithful to the tissues from which they were obtained.

The neuron somas of the spinal cord, cerebellar, and cerebral cultures do not migrate out of the original explant area. Their processes however, may extend for considerable distances into the outgrowth zones. Characteristic central neurons become visible

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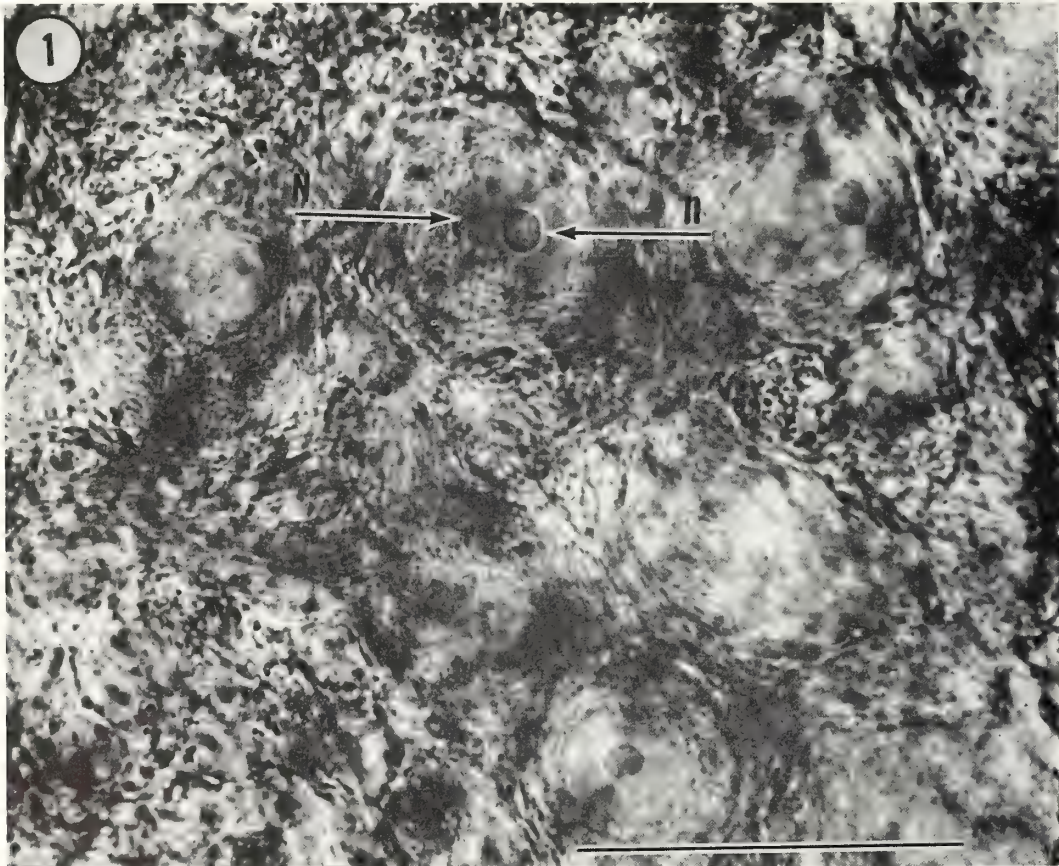
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within the first 4 to 10 days of culture and remain so for their entire in vitro life, which may be prolonged for times ranging up to 6 months. Their size may vary considerably from the largest with diameters of more than 50μ as seen in the spinal cord (fig. 1), through the $25\text{--}35\mu$ diameters of some cerebellar neurons (fig. 7) to the small ($15\text{--}20\mu$) diameters of the cortical neurons during their first weeks of culture (fig. 9). The neuronal population of cultured cerebral neocortex also duplicates in vivo developments (20) by undergoing a gradual increase in cell diameters as the packing density decreases and interneuronal growth of neuropil increases (fig. 10).

The peripheral neurons of the dorsal root ganglia are not obscured by surrounding neuroglia and can be identified within a day of explantation (fig. 3). They

are also characterized by the presence of satellite cells closely applied to their perimeters. In time, cultured neurons develop characteristic Nissl patterns which may be visualized either in the living or fixed and stained state (2, 3, 12, 13, 17). Various kinds of silver impregnations have also been employed to identify and characterize cultured neurons (2, 17, 18).

The neuroglia remaining within the area of the original explant are not easily characterized as astrocytes or oligodendroglia in the living state, since their cell boundaries tend to be lost within the surrounding mass of tissue. Generally, their nuclei are smaller than those of neighboring neurons (fig. 7) and thus permit simple identification. Most previous studies of neuroglia (15, 16, 19, 21) have stressed the more easily observed cells found either in the outgrowth zones or

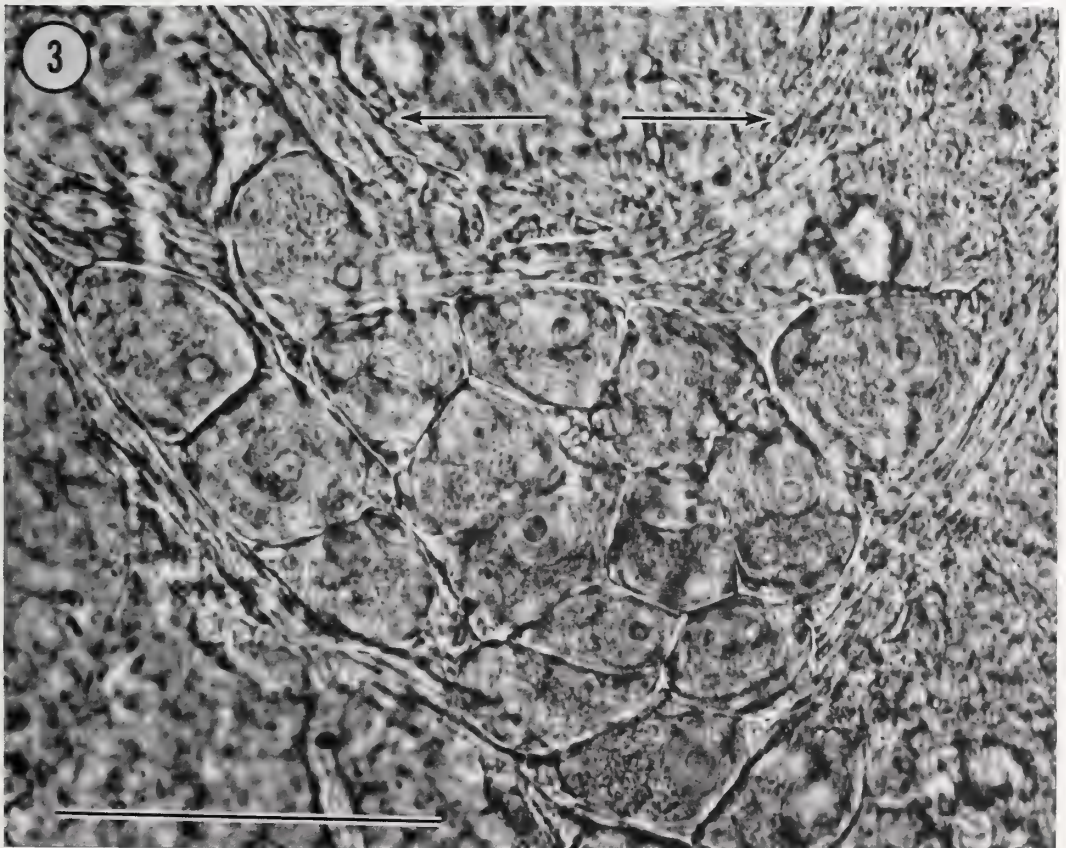
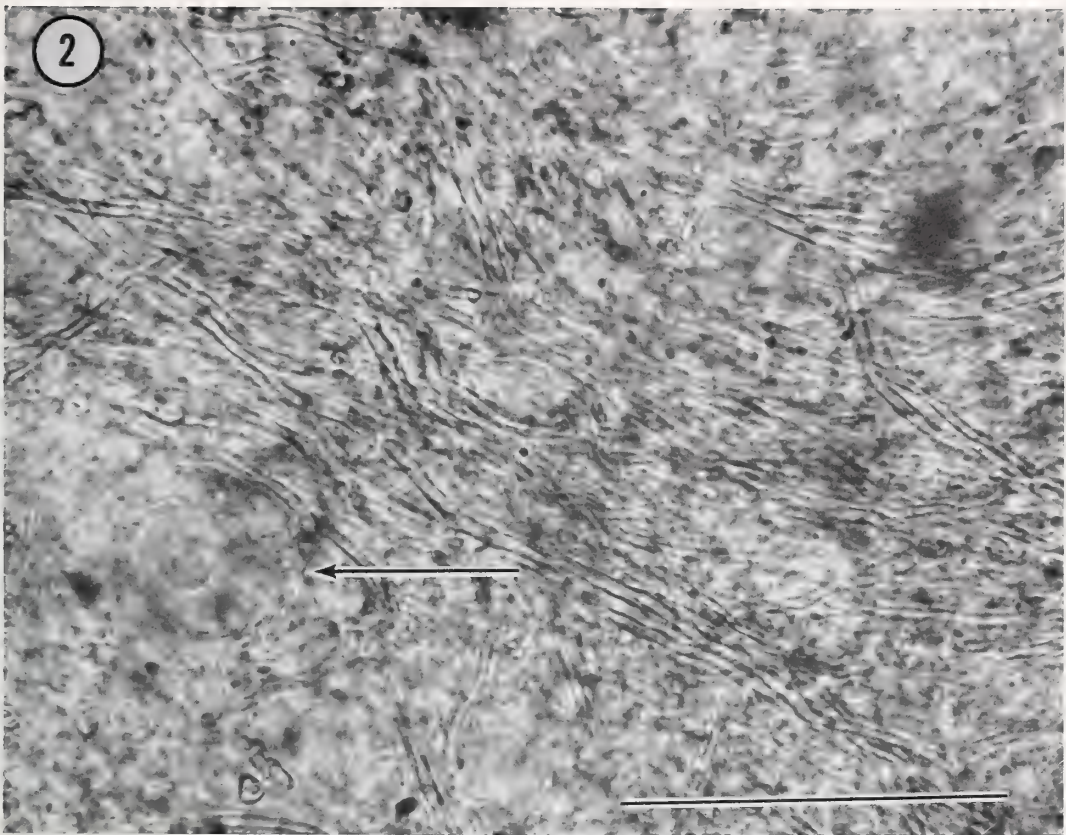


FIGURES 1 to 11. All photographs were taken of living, unstained cultures at magnification of 600 diameters. Unless otherwise specified, lighting was obtained by ordinary bright-field illumination, and the scale represents 100 microns.

FIGURE 1.—17-day-old mouse embryo spinal cord after 14 days in vitro (DIV). Arrow "N" designates a nucleus and arrow "n" a nucleolus.

FIGURE 2.—12-day-old mouse embryo spinal cord-striate muscle fragment. 28 DIV. Myelinated axons in the spinal cord area and a single neuron perikaryon (arrow) in focus.

FIGURE 3.—12-day-old mouse embryo spinal cord-striate muscle fragment. 23 DIV. Spinal ganglion (peripheral) neuron somas and their entering or leaving axons (arrows)



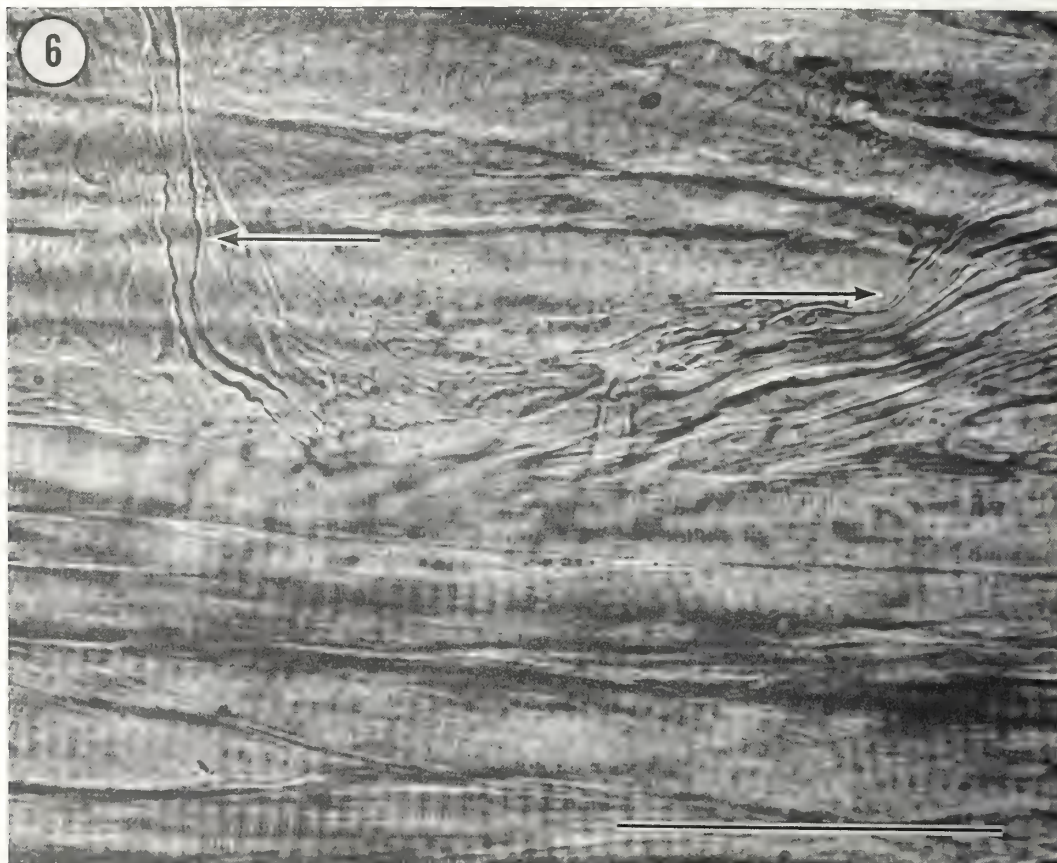
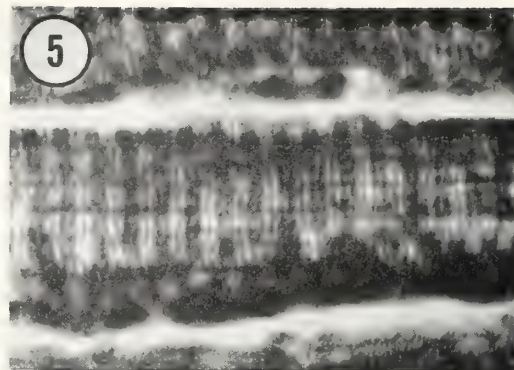
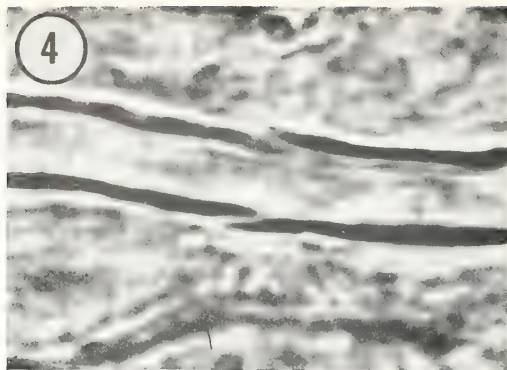


FIGURE 4.—13-day-old mouse embryo spinal cord-striate muscle fragment. 24 DIV. A myelinated peripheral axon with a cleft of Schmidt-Lantermann. Scale=30 microns. Bright-field illumination.

FIGURE 5.—12-day-old mouse embryo spinal cord-striate muscle fragment. 31 DIV. A single muscle fiber showing characteristic cross striations. Scale=50 microns. Phase contrast.

FIGURE 6.—13-day-old mouse embryo spinal cord-striate muscle fragment. 22 DIV. Myelinated axons (arrows) approach and enter an area composed principally of striate muscle fibers. See legend to figure 1.

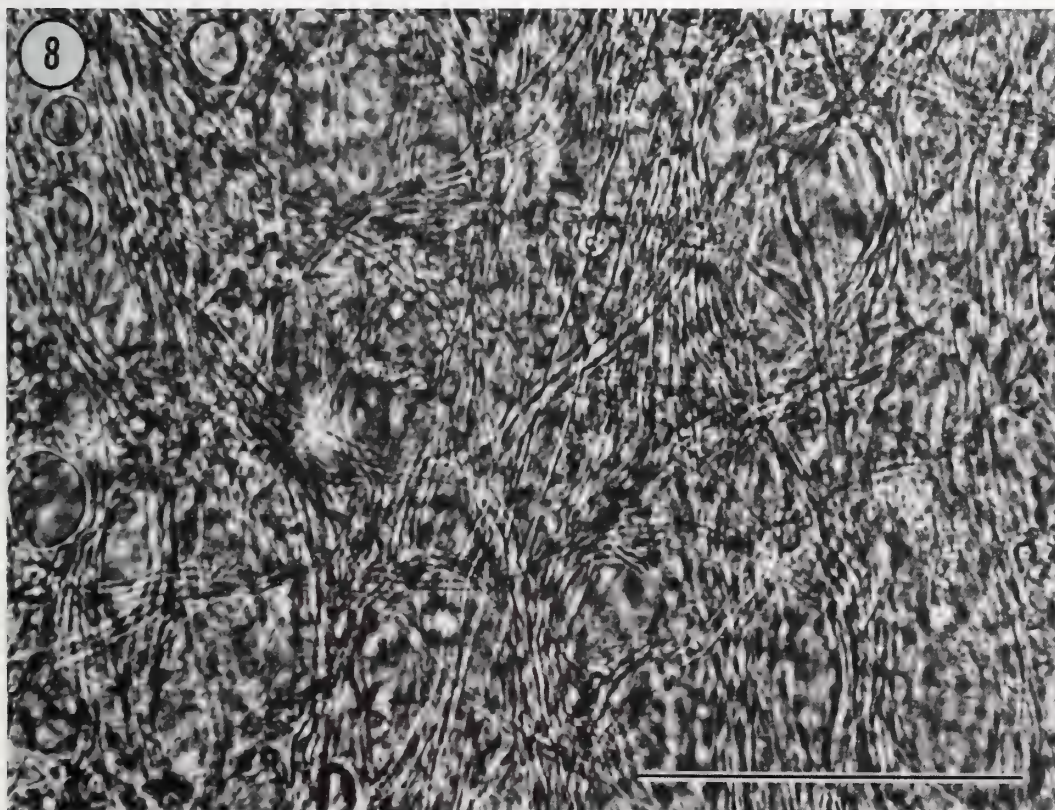
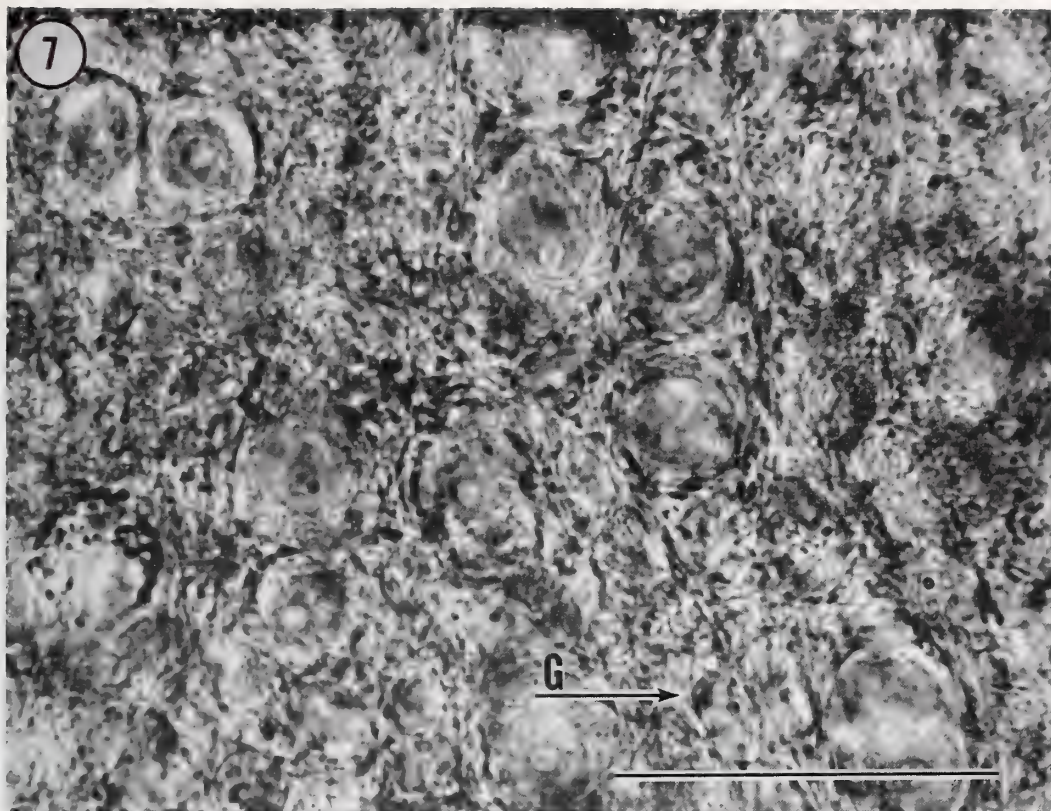


FIGURE 7.—Newborn mouse cerebellum. 15 DIV. Neuron somas. An arrow “G” designates a neuroglial cell for contrast. See legend to figure 1.

FIGURE 8.—Newborn mouse cerebellum. 21 DIV. Myelinated axons. See legend to figure 1.

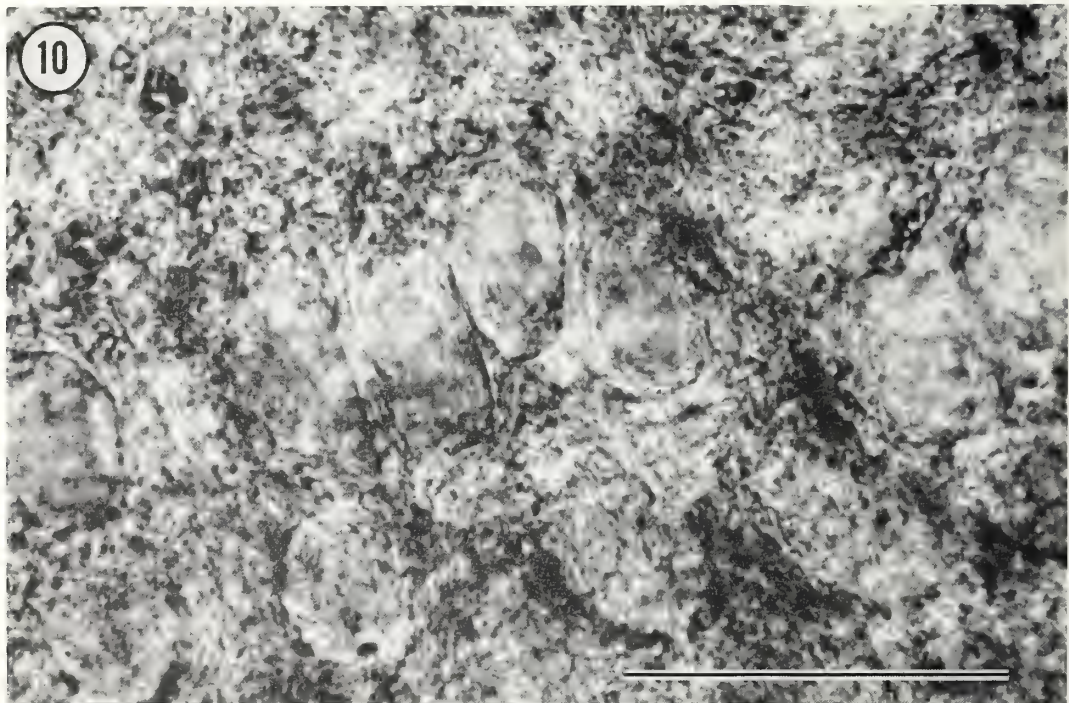
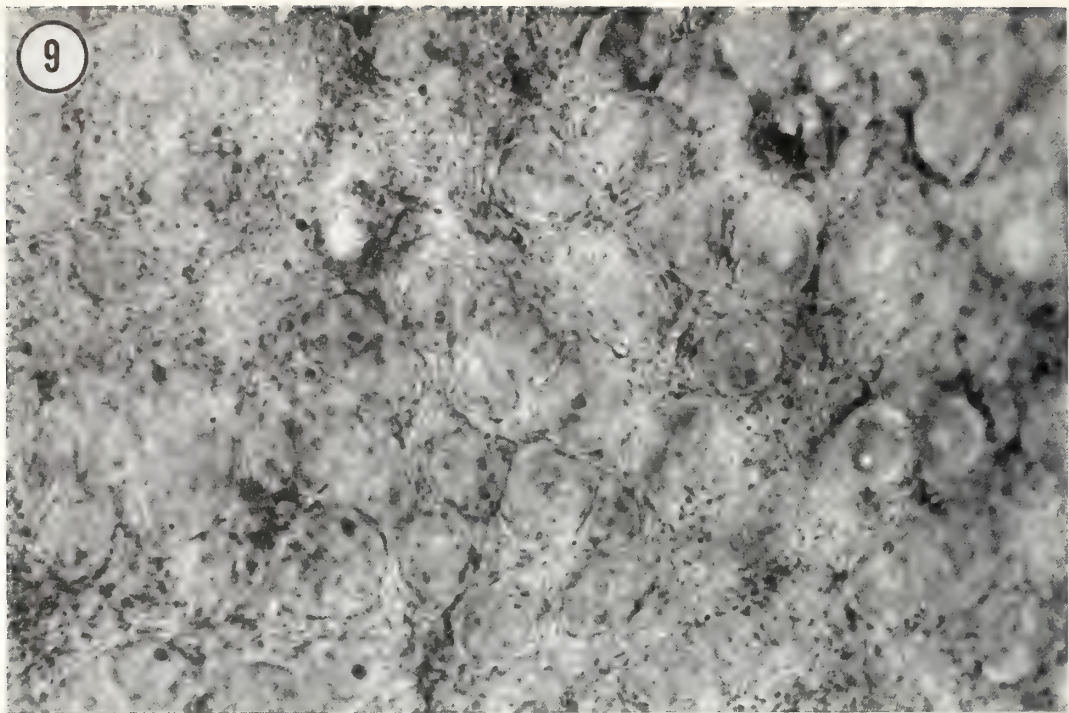


FIGURE 9.—2-day-old mouse cerebral neocortex. 20 DIV. Densely packed neuron cell bodies. See legend to figure 1.

FIGURE 10.—1-day-old mouse cerebral neocortex. 90 DIV. Neuron somas which are larger and separated by a dense neuropil. See legend to figure 1.

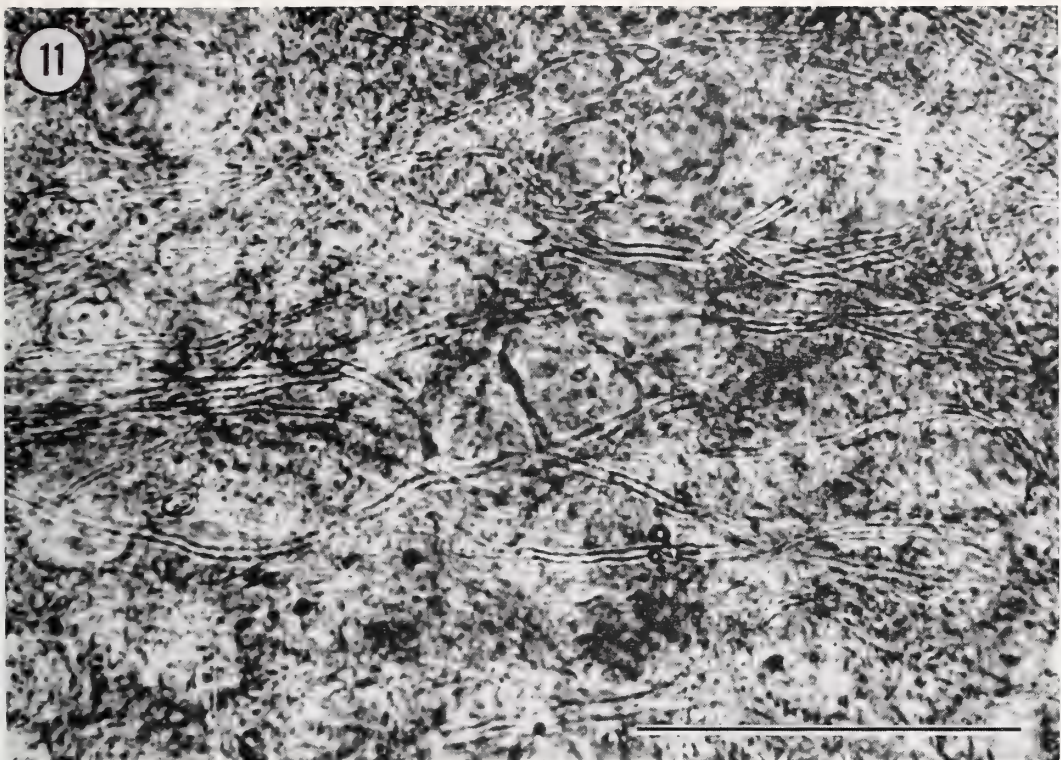


FIGURE 11.—3-day-old mouse cerebral neocortex. 23 DIV. Myelinated axons. See legend to figure 1.

in markedly flattened areas of the original explant. These have revealed varieties of structure which permit some classifications into oligodendroglial and astrocytic forms. The application of more recently developed techniques of histochemistry, microchemistry and electron microscopy should lead to an extension of previous classifications into metabolic and functional types.

Myelin formation, one of the functions of neuroglia and Schwann cells, has been observed in these (figs. 2, 4, 6, 8, 11) as well as other tissues (3, 6, 14, 17). In our laboratory, the time of myelination is shortest in the spinal cord, the earliest appearance having been recorded after 5 days in vitro (DIV). Cerebellar myelin usually becomes apparent during the second week. The cerebrum usually produces its scanty myelin by the third week in vitro. The distribution of myelinated axons is also somewhat different depending on the tissue involved. Myelinated axons of spinal cord frequently form a rather dense tract surrounding the fragment. They may also extend into the outgrowth zone for long distances (1 to 3 millimeters) while maintaining their myelin sheaths. These central axons can be readily differentiated from peripheral myelinated axons of the spinal ganglia which may be present in the same preparation. The latter are usually thicker

axons with heavier myelin sheaths, possessing characteristic Schwannian nuclei tightly adherent at a number of locations along their length, and have an abundance of characteristic nodes of Ranvier (rare in central myelinated axons) and clefts of Schmidt-Lantermann (fig. 4). Myelinated axons may also extend for long distances from the cut under surface of neocortical fragments. Should subcortical neurons be included in the preparations, myelinated axons may course from these areas to the cortex. Myelinated axons have now been observed in the outgrowth zone surrounding cerebellum, contrary to our earlier report (Bornstein and Murray, (3)), but they rarely extend beyond the dense collar of neuroglial surround.

The participation of the neuroglial cell in the formation of central myelin in vitro was denied by Hild (14) but inferred from our serial observations of kitten and rat cerebellum (22) and fully confirmed by the ultrastructural details revealed by Ross's electron micrographs of the mouse and rat cerebellum (6).

The fact that a cultured community of cells from the nervous system develops significant intercellular relationships was thus established in this instance of the glial (Schwannian)-neuronal interaction to produce myelin. The staining characteristics (Bodian, Holmes) of the neurons (2, 18) as well as the appearance of the

classical (23) image of synapses in electron micrographs (2, 24) led us to the belief that significant interneuronal relationships might also be developing among the cultured neurons. Crain's studies of cultured spinal cord (25, 26), cerebral neocortex (8), and spinal cord-muscle preparations (27) leave no doubt that complex bioelectric activities, the result of neuronal interrelationships, are developed and maintained in long-term cultures of mammalian nerve tissues.

Since the main theme of this conference is concerned with manifestations of virus infections, you probably would wish to know whether or not the cultured tissues may be used to explore problems relating to disease processes. Time does not permit a detailed presentation of the various patterned reactions cultured nerve tissue may reveal on exposure to antimetabolites (Yonezawa, personal communication), heavy metals and bacterial exotoxins (28) and various other manipulations of their environments. We have published a series of studies related to the demyelinating diseases (1, 29, 30, 31) and Winkler has extended the observations to peripheral nerve cultures (32). These are proving of some value in the amplifications of previous knowledge obtained by more classical techniques in the analysis and understanding of the phenomena in terms of a possible auto-immune mechanism.

It follows that significant information may be obtained when these model systems are exposed to the influences of various virus agents.

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DISCUSSION

KOPROWSKI: Would you comment about the role of the cell-bound antibody and the 19-S and 7-S components of the serum antibody in the demyelinating process observed in your tissue culture system.

BORNSTEIN: We have not studied cell-bound components, but I do not doubt at all that cells produce antibodies. Winkler, at Harvard, has demonstrated a demyelinating action with serum and lymphoid cells obtained from peripheral lymph nodes, when both are from animals with experimental allergic neuritis. However, the serum may be positive and the nodes negative, or vice versa, but they produce the same pattern of change on the cultures of periph-

eral nerve (trigeminal) tissue. In our experiments, demyelinating pattern was produced by the 7-S fraction of the three rabbit sera analyzed. It was never demonstrated by the 19-S fraction.

KIES: Do you think the 19-S is the suppressing antibody?

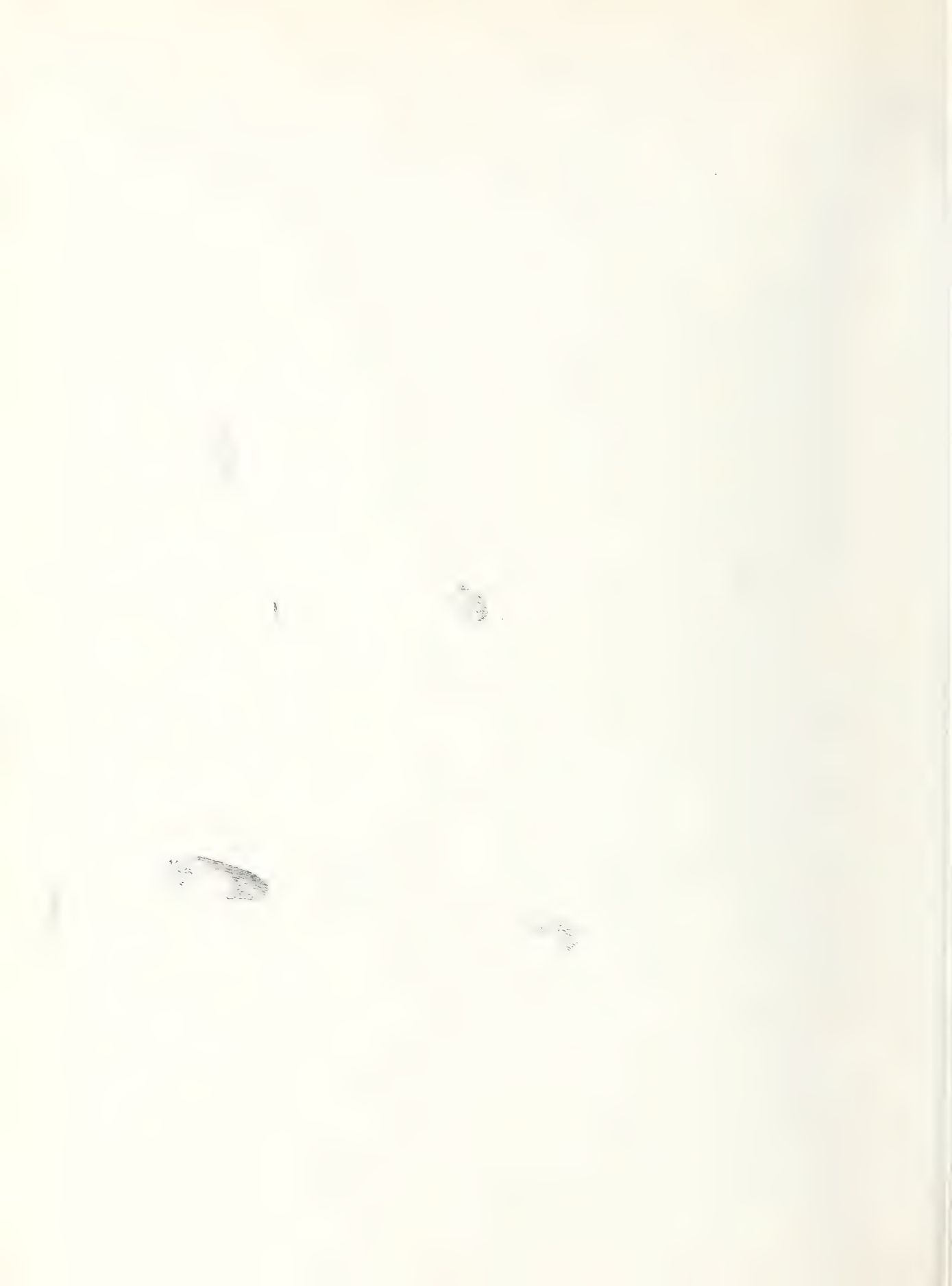
BORNSTEIN: According to Dr. Paterson, the 19-S is a suppressing antibody.

PATERSON: We had an occasional animal that had no demyelinating effect in your system but had high titer 19-S CF antibody. This shows that 19-S antibody does not produce toxic effects and this is consistent with the protective effect. The crucial experiment for us to do is to put 19-S in first, and then follow with 7-S.

BORNSTEIN: One thing I may mention is that Dr. Paterson supplied us with nine sera from animals which had received lymphoid cells from affected animals. Three of those animals had developed demyelinating disease by passive transfer. The sera were examined "blindly" and, from the effect on the cultures, we disclosed two of the three that had developed the disease by passive transfer.

FIELD: I believe that you were surprised to find so many amyotrophic lateral sclerosis sera showing a demyelination effect on your cultures. I am not worried about demyelination, which certainly occurs, but I am worried about specificity. Here you have definite destruction of myelin and once again one is faced with the possibility that this is the cart and not the horse, and that these antibodies are formed as a result of primary myelin destruction.

BORNSTEIN: We have tested the serum of some 30-odd patients with destructive, but not demyelinating, lesions of the nervous system: degenerative, vascular, postoperative, etc., who probably have destroyed myelin or products of destroyed myelin floating around in their bodies; yet a very small percentage compared to the others has demonstrated these anti-myelin factors as far as the tissue culture system is concerned.



Some Observations on the Clinical Immunology of Multiple Sclerosis

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Since multiple sclerosis has been widely accepted as an "auto-immune" disease (1) it is important to ascertain how far this view is supported by the clinical immunology of the condition. Several workers have investigated the complement fixing antibodies of multiple sclerosis using brain or brain extracts as antigens and whilst some have found such antibodies (2, 3, 4) others have not been able to do so (5, 6, 7, 8); or have reported them to occur only infrequently (9). Reasons for these differences have been considered by Macleod et al. (10, 11) who like Goudie et al. (12) have pointed out the importance of the complement level at which tests are carried out. The discrepancies are also reviewed at some length by McAlpine et al. (13). Our own group in Newcastle has not been persuaded that an adequate observational basis yet exists for accepting MS as essentially an auto-immune disease and has directed its efforts to glean further information especially with the use of the purer antigens which have recently become available. In particular, we thought it important to find out how far agglutinating antibody to brain or its derivatives (e.g., encephalitogenic factor—EF) was present specifically in the serum of MS patients and to what extent such antibodies also occurred in other conditions believed to have an immunopathological basis—notably Hashimoto's thyroiditis or rheumatoid arthritis. At the same time the presence of antibody against kidney was also investigated (cf. Gajdusek (14)).

MATERIAL AND METHODS

Sera were obtained from patients with florid MS, Hashimoto's thyroiditis and rheumatoid arthritis as well as from students and nurses immunized six weeks previously with B.C.G. Normal sera were obtained at donor sessions of the Newcastle Blood Transfusion

Service and were subject to the customary checks (Wassermann reaction, etc.). In general, sera were stored at -20° C. for not longer than 3 to 4 weeks before use.

Sera were obtained by cardiac puncture from guinea pigs with experimental allergic encephalomyelitis resulting from the inoculation of human brain with Freund's adjuvants.

For brain or kidney suspensions or extracts, a slight modification of the bis-diazo-benzidine method of coating red cells (15, 16) was found satisfactory using human group O, Rh+ red blood cells. Freshly prepared reagents were essential for the success and consistency of the method. Details are given by Caspary et al (17).

For determining agglutinating type antibody to EF the tanning method of Boyden was used (18).

The effect of EF upon platelet adhesiveness to glass when added to MS plasma was tested by a modification of Helen Payling Wright's method (19, 20). Further details are set out by Caspary (21).

Four patients suffering from multiple sclerosis have been tested with intracutaneous inoculations of EF in saline ($1\mu\text{g}$) on the volar aspect of the forearm. Control injections with saline alone and an encephalitogenically inactive saline extract of brain were made simultaneously.

RESULTS

Agglutinating Antibody Titres

Table I (A and B) sets out antibody titres to brain and kidney suspensions found in the serum of the cases indicated. It will be noted that whilst agglutinating antibody to brain is raised in all four abnormal state studied, the titres in MS were distinctly lowest. Whilst they were probably significantly raised as compared

TABLE IA.—Antibodies to Brain and Kidney Suspensions in Serum of Patients Suffering From Multiple Sclerosis, Hashimoto's Disease, or Rheumatoid Arthritis, and in Normal Individuals Recently Immunized With B.C.G.

	Number of cases	Antigens			
		Brain		Kidney	
		Mean titre	Significance (P value)	Mean titre	Significance (P value)
Normal controls	32	0.50±0.75		0.50±0.75	
Multiple sclerosis	52	1.06±0.82	0.02–0.01	0.70±0.74	0.3–0.2
Hashimoto's disease	32	1.75±1.60	0.001	0.72±0.83	0.3–0.2
Rheumatoid arthritis	17	1.76±0.81	0.001	0.63±0.86	0.6–0.5
B.C.G. immunization	16	2.63±2.28	0.001	1.00±1.54	0.2–0.1

TABLE IB.—Comparison of Circulating Antibody Titres Between Diseases

A	B	Significance (P value)
Multiple sclerosis	Hashimoto's disease	0.02–0.05
Multiple sclerosis	Rheumatoid arthritis	0.02–0.05
Hashimoto's disease	Rheumatoid arthritis	0.9
Multiple sclerosis	B.C.G. immunization	0.01
Hashimoto's disease	B.C.G. immunization	0.2–0.1

with normal subjects ($P=0.02-0.01$) they were possibly significantly lower than in either Hashimoto's disease or rheumatoid arthritis ($P=0.05-0.02$). On the other hand brain antibody titre after B.C.G. immunization was significantly higher than in MS ($P=0.01$).

In none of the conditions examined was the titre of antibody to human kidney raised. On the other hand animals which had been given encephalitis by the inoculation of human brain and Freund's adjuvant showed markedly raised titres against human (group O) brain and kidney and also against guinea pig brain (table II). In preliminary experiments in which sim-

TABLE II.—Circulating Antibodies to Tissue Suspensions by the Bis-Diazo-Benzidine Method

	Mean titre		Significance (P value)
	Normal guinea pigs	Encephalitic guinea pigs	
Human brain antigen.	0.95±1.12	3.81±1.94	<0.001
Guinea pig brain antigen.	1.15±1.15	3.76±1.93	<0.001
Human kidney antigen.	0.84±0.7	4.53±2.99	<0.001
Comparison of titres between the antigens in encephalitic animals:			
Human brain antigen with guinea pig brain antigen. $P=0.9$			
Human brain antigen with human kidney antigen. $P=0.4$			

ple tanning of red blood cells was tried out for these tests no results could be obtained and this, it was felt, ruled out the possibility of positive results with human kidney being simply due to human serum protein contaminants since previous work had shown that human serum albumin may be readily fixed to the surface of erythrocytes by the ordinary tanning procedure. The positive results with kidney antigen are therefore acceptable as genuine.

Experiments with EF

(a) Turning to the results obtained by the use of highly purified encephalitogenic factor (EF) it is seen that whilst MS (both acute and chronic) shows a significantly increased number of sera with agglutinating antibody this is not the case with Hashimoto's disease or rheumatoid arthritis (table III). Other conditions in which considerable destruction of nervous tissue may be presumed to have occurred also gave a high number of positive sera and so did B.C.G. immunization.

(b) EF (100 μg in a total volume of 1.5 ml.) has been found to cause slight but definite and repro-

TABLE III

Subjects	Serum			Significance against normal (χ^2 test)
	Positive	Negative	Total	
Normal	9	35	44	
Acute M.S.	11	7	18	<0.001
Chronic M.S.	27	20	47	<0.001
G.P.I.	62	38	100	<0.001
Neurological disease other than M.S. or G.P.I.	18	14	32	<0.001
Huntington's chorea	6	10	16	0.1
Presenile dementia	9	5	14	<0.001
Hashimoto's disease	5	14	19	0.8–0.9
Rheumatoid arthritis	7	10	17	0.1–0.2
B.C.G. subjects	17	14	31	0.02–0.01



FIGURE 1. Intradermal reactivity test (at 24 hours) on guinea pig 15 days after inoculation with human brain and Freund's adjuvant (2 days after onset of mild signs of allergic encephalitis). On the right encephalitogenically inactive saline extract of brain has been injected; above 0.02 μ g EF (in saline); on the left 0.05 μ g; and below 1 μ g EF. The central points of the injection sites were marked with India ink. Volume injected was 0.1 ml in each case.

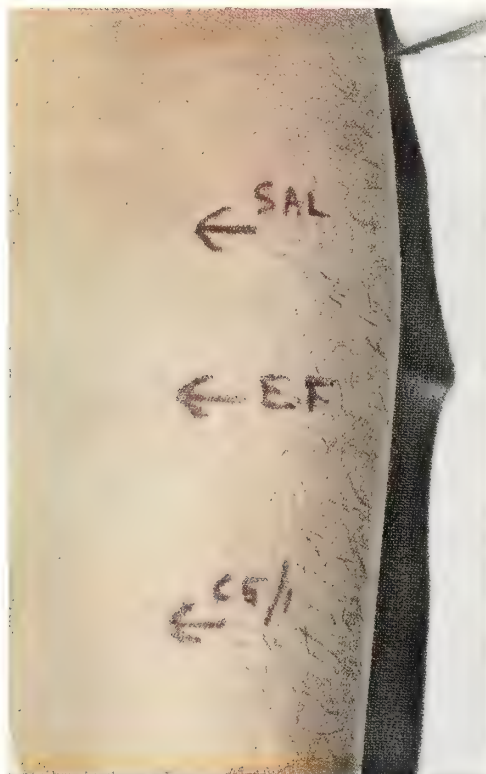


FIGURE 2. Intradermal reactivity test (at 24 hours) in volar aspect of forearm of a chronic MS patient. Arrows point to site of saline injection (above); 1 μ g EF (middle); and encephalitogenically inactive saline brain extract (below). Volume injected was 0.1 ml in each case. The result was negative in each case.

ducible increased adhesiveness of platelets to glass (20). Whether there is also some degree of platelet lysis is under investigation.

(c) Neither in guinea pigs (fig. 1) nor in humans (fig. 2) was skin delayed (tuberculin) type sensitivity demonstrable to intracutaneous inoculation of EF. That the guinea pigs with EAE had indeed become sensitized to the heterologous (human) material injected is shown by the marked reaction to the encephalitogenically inactive aqueous brain extract tested (right hand inoculation site in figure 1). At the three other inoculation points (marked by india ink dots) 0.02, .05 and 1.0 μ g EF had been inoculated in saline 24 hours previously.

The negative result in a patient suffering from chronic MS when 1 μ g EF was injected into the skin of the forearm is seen in figure 2 where it can also be seen that there is no sensitivity to the encephalitogenically inactive aqueous brain extract. In other tests when as much as 100 μ EF was inoculated no appreciable reaction greater than that in a control test was found after 24 hours.

DISCUSSION

The finding that antibody to brain was produced in higher titre in the non-MS conditions than in MS itself was surprising. Hashimoto's disease, generally accepted as a model of an "auto-immune" condition, was on the border line of difference from MS ($P=0.02-0.05$), i.e., it appeared to show significantly higher titre to brain than did MS itself. This phenomenon may be related to the known propensity of Hashimoto cases to show gastric microsomal antibodies (22) and raises the problem once again of the significance of these antibodies in the production of "auto-immune disease." The question must be raised whether subjects with thyrotoxicosis are as a result able to produce "auto-antibodies" to a variety of tissues, and has been discussed in some measure by Anderson et al. (22).

For a disease supposedly due to "auto-immunization" with brain, MS showed remarkably little agglutinating antibody and the results set out above offer little support for the thesis that the condition has a primary immunopathological pathogenesis. Furthermore the study with EF as antigen suggests strongly that antibody to this factor arises whenever there has been nervous tissue disintegration, i.e., that it is a consequence rather than a causal factor in the disease. In fact the evidence that MS is primarily immunological in pathogenesis is not strong when examined critically. It is, indeed, interesting to note that even

amongst conditions commonly accepted as having an "auto-immune" basis it has been claimed that "with the possible exception of certain blood dyscrasias, no pathological process has been shown to be the result of autoimmunity; the evidence is perhaps strongest, but still not conclusive in relation to chronic thyroiditis" (22).

At least two reports have, however, appeared indicating that the human brain may show a severe immunological response to the parenteral injection of foreign nervous tissue. In 1957 Uchimura and Shiraki (23) published their findings in the brains of patients who had died from neuroparalytic accidents in the course of suspected rabies treatment. These changes were presumed to result from an allergic response to the foreign brain material inoculated, "although an auxiliary role may be played by a biochemical component of the killed virus" (24). Uchimura and Shiraki's work has been regarded as "the most convincing argument" for the close relationship between EAE and MS (25) and so for the fundamentally immunological pathogenesis of MS. Whilst the pathological picture in these cases of neuroparalytic accident are certainly very similar to multiple sclerosis there are some features which suggest that too close an analogy between the two conditions should not be drawn. Thus Lumsden (26) counts himself as "one of the few who do not accept Shiraki's cases as even acute MS—chiefly on the grounds that the inflammation and oedema are disproportionately great to the demyelination and lipid breakdown and that the lesions are evidently monophasic and nonprogressive peripherally." However, this author is of the opinion that a neuro-allergic mechanism in man "could go a long way to producing the morphological peculiarities of MS" and that "it * * * (is) * * * hard * * * to doubt that allergy * * * seems likely to be * * * the factor which triggers off the process in the new plaque * * *" (loc. cit.). This is not the place to consider these pathological similarities more extensively. Whilst it must be admitted that the end picture in Shiraki's cases has some closer resemblances to MS, it should not be accepted, however, that this picture could not arise as a sequel to a single explosive event quite unlike multiple sclerosis in its natural history.

Jellinger and Seitelberger (27), too, have published evidence that the human may react to repeated inoculations of foreign brain material in much the same way as do lower animals.

In the author's opinion neither Shiraki's nor Jellinger's findings prove more than the ability to produce

acute lesions in human brain by methods similar to those known to be effective in laboratory animals. The author would agree with Lumsden (cited above) that allergy most probably plays a part in the evolution of plaques though not in the context in which Lumsden suggests but rather in the manner described below.

The rise in gamma globulin content of the spinal fluid in MS has also been held to be potentially important evidence that an immune process is at work in the evolution of the disease. Unfortunately the immunological character of this γ globulin (discussed at length by McAlpine et al. (13)), is still not clear. Few investigations of this fundamental point have been made, but Field and Ridley (28) concluded that "no great local formation of antibody to brain tissue" could be demonstrated by the Steffen technique they used. More recently Caspary (29) has failed to demonstrate any immunological difference between γ globulin of serum, normal spinal fluid and MS spinal fluid using an agar diffusion technique. Obviously a good deal more work is required to elucidate the nature of the γ globulin increase found in MS (and in neurosyphilis) before it can be reasonably held to support the view that an auto-immune allergic process is at work within the central nervous system.

The idea that MS is associated with minute thrombi in small blood vessels of the nervous system is an old one and goes back in fact to Rindfleisch (1863) and Ribbert (1882) (30, 31). Its foremost protagonist in recent years has been Putnam (32, 33), who indeed published photomicrographs showing platelet thrombi in recent lesions. During the last 5 years Courville (34) has also favored the idea and has remarked upon the resemblance between the lesions found in fat embolism and those in multiple sclerosis. If some mechanism could be elucidated whereby small veins (and not small arteries as in Courville's fat embolism) could be blocked in a potentially reversible manner then this might serve as a possible pathogenetic basis for MS. If such small blockages were to persist for only a few hours, then one could suppose that reversible interference with function of the surrounding nervous tissue would take place and when the plug resolved there would be a *restitutio ad integrum*. If, however, such a plug persisted—shall we say 72 hours—then some degree of permanent structural damage might ensue.

Consideration must be given to the negative findings with regard to skin sensitivity both in EAE and in MS. It is well known that guinea pigs made encephalitic with homologous brain and Freund's adjuvant do not show appreciable skin reactivity when tested with the original brain material. It can be said at

the outset, therefore, that skin reactivity is not an essential concomitant of the allergic response which manifests itself as EAE.

Recently Alvord and his collaborators (35) have claimed that skin tests carried out before the onset of signs of EAE give clearer positive results in guinea pigs than after the appearance of signs. They recommend, moreover, larger inocula in carrying out these tests, though these carry with them the risk of introducing appreciable amounts of nonencephalitogenic contaminant which may be dermally active.

From the failure of human MS patients to give a positive skin reaction with EF it cannot be concluded that immunization against EF has not occurred in the disease. Thus we cannot be sure that skin reactivity necessarily mirrors that of the central nervous system and indeed O'Grady (36) has brought forward some evidence that skin reactivity may be divorced from that of internal organs. Moreover the absence of skin reactivity in guinea pigs inoculated with homologous brain, referred to above, indicates that an undoubted delayed hypersensitivity response in the nervous system may be unaccompanied under certain conditions by skin reactivity.

The above arguments would indicate that there is no sure evidence that an immune response forms the primary pathogenetic basis for the development of multiple sclerosis. The hypothesis of platelet microagglutinates or venous endothelial paving by platelets outlined above offers an attractive working hypothesis for the continuing recurrent episodes of varying intensity met with in multiple sclerosis. Further experiment will be needed to assess the phenomenon of increased platelet adhesiveness in the presence of EF but it should be emphasized that the difference it induces is small and not of the order encountered in thrombotic or purpuric diseases.

All this leaves open the question of what causes the primary lesion(s) in the brain from which it is suggested EF may be resorbed into the small veins or capillaries. Now a certain number of "normal" people appear to have antibody to EF already in their blood, and it may be it can arise in response to some non-nervous antigen such as B.C.G. (18). But in most cases the author would suggest that multiple sclerosis might very well be due to some infectious agent which results in primary lesion(s) and that the platelet agglutinating mechanism is one by which the disease chiefly continues its course. Evidence that some transmissible agency can produce changes in sheep and goats is presented elsewhere in the proceedings of this workshop-symposium. Should these latter observations be

confirmed and extended then a picture of multiple sclerosis might emerge as a "slow virus infection" in the first place, with progressive episodic extension of pathological changes by the platelet agglutinate mechanism formulated above.

ACKNOWLEDGMENTS

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SUMMARY

Some of the clinical immunological phenomena of multiple sclerosis are examined and the position of the disease as an "auto-immune" condition assessed. Little direct evidence exists. On the other hand data are presented suggesting that continuing episodes of the illness may have an allergic basis and it is suggested this may be mediated through platelet agglutination whilst the primary condition may well be a slow virus infection.

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DISCUSSION

POSKANZER: I was very excited when Dr. Field told me about this test because we have no measure of activity in multiple sclerosis and it would be a very useful tool. The only indication of activity now available is the appearance of lymphocytes in the spinal fluid; all the other tests such as the gold sol curve do not help us as indicators of the activity of the disease. We tried to reproduce his results but I am afraid we could not duplicate them. It is probably our own incompetence, but one of the things

that worried me about the test in the first place is that the platelet count itself is not generally accurate to within 10 percent, and is not reproducible, at least in our hands.

FIELD: May I say that Mr. Caspary does his counts absolutely blind, without being told what they are, though it is true that he has had a great deal of experience in counting platelets. I admit that platelet counting is normally a very uncertain business and we are hoping to tackle this by a radioactive method in the near future.

MARGOLIS: To me, one of the most interesting developments in the field of inflammation is the experimentation showing that the changes in permeability of blood vessels are at the venular level, rather than capillary level. This might help explain the perivenous localization of all these reactions, and relate sludging and platelet microthrombi to a change in small vessels. Then there is another observation. Rarely, in some of these inflammatory allergic diseases, we see a localization which is very peculiar, namely subpial and subventricular demyelination. Can you comment on these observations?

FIELD: It has been suggested that there is some thrombocyte-agglutinating material in the cerebrospinal fluid in multiple sclerotic cases, and certainly, looking at some cases of the disease, one is struck by a seam of demyelination on the surface of the pons and in the angles of the lateral ventricle in contact with cerebrospinal fluid. As far as the venous endothelium is concerned, what I had in mind was a sort of sticking or pavingmenting of the platelets over the endothelium, for quite some distance, and this might prevent easy resorption of the cerebrospinal fluid. If that were to persist for, shall we say, 24 hours and then the platelets were to unstick, there would be no lesion formed at all. But if it were to persist for 3 or 4 days, then organic damage, demyelination, around the vein might ensue. The importance of the veins is borne out by the work on allergic phenomena in the rabbit omentum in which it is shown that the changes are centered on the small veins.

The Virology, Pathology and Epizootiology of Scrapie

Chairman

I. H. PATTISON

Viral Characteristics of the Scrapie Agent in Mice

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INTRODUCTION

During the first part of this workshop-symposium we have been mainly concerned with a detailed analysis of the epidemiological, clinical, and neuropathological data available on progressively degenerative diseases which have as their final targets the nervous system of the host. Because of their unknown etiologies they are placed in the category of diseases in search of viruses. Although, as described earlier in these sessions, our major laboratory program is directed toward the establishment of an infectious, serially transmissible, viral agent in the etiology of these diseases, i.e., kuru, amyotrophic lateral sclerosis, subacute sclerosing leucoencephalitis, multiple sclerosis, Dawson's inclusion body encephalitis, etc., the working models that are providing significant information on the existence and nature of "slow-growing" viruses affecting the central nervous system are scrapie and visna virus diseases of sheep, Aleutian disease of mink, and lymphocytic choriomeningitis infections of mice. Scrapie, as a clinical entity, has been recognized in sheep in England since as early as 1732, and in other European countries, Spain, Germany, and France, it has been known since the late 18th and early 19th centuries. Indeed, one need only consult the extensive bibliography on scrapie (1) included in this publication to follow the development of our knowledge of this disease. However, it was not until relatively recently that investigators reported the successful experimental transmission of the disease in sheep by injecting healthy sheep with suspensions of the brain and spinal cord of animals affected with scrapie (2, 3, 4). More recently an encephalopathy has been induced and serially transmitted in mice following their inoculation with the brain and spinal cord of naturally

infected sheep and experimentally infected goats (5, 6). Properties of the scrapie agent and its behavior in mice have been described by Morris and Gajdusek (6), Eklund, Hadlow and Kennedy (7, 8), and Gibbs, Gajdusek and Morris (9).

We have now obtained: (1) confirmation of Chandler's (5) observation on the thermostability of the scrapie agent as compared to most other viruses; (2) evidence for a moderate ether sensitivity of the agent; (3) estimate of the size of the scrapie agent by gradacol membrane filtration; (4) further data on the relative susceptibility to the scrapie agent of male and female mice; and (5) observations on the influence of the route of inoculation on the experimental transmission of the disease to mice. We also report further attempts to demonstrate antibody or an immune state in scrapie, specifically: (1) to detect antibody by neutralization, complement-fixation, precipitation, or fluorescent antibody techniques, using mouse brain antigen and sera from animals after attempted immunization with the mouse scrapie agent; and (2) to determine whether an immune tolerant state occurs in mice inoculated during the neonatal period. Finally, we report the production of scrapie-like encephalopathy in mice by inoculation of serum from a natural case of scrapie in a ram and from an additional seven naturally occurring cases of scrapie in American sheep by inoculation of brain tissue.

It should be noted that we have not demonstrated that the sheep scrapie virus is the etiological agent of the illness developing in mice several months after inoculation of scrapie sheep brain or serum, or scrapie goat brain, or of affected mouse brain in passage series. However, the signs of illness and the neurohistological lesions in affected mice are remarkably similar to those

seen in scrapie affected sheep and goats. The neuropathological lesions observed in our experimental mice will be discussed by Mrs. Elisabeth Beck (10).

MATERIALS AND METHODS

Study Material.—The infectious materials employed were brain tissue obtained from one goat experimentally infected with the sheep scrapie agent and from eight sheep with naturally acquired scrapie. The goat brain material was obtained from Mr. I. Pattison, Agricultural Research Council Field Station, Compton, Berkshire, England. The Compton goat brain has been shown to contain the scrapie agent in tests in both goats and mice and represented the ninth passage in goats of the infectious agent since its recovery from the brain of a sheep; this strain of scrapie has been designated Compton SPG-9. The sheep serum and brains were obtained from various geographical areas of the United States through the courtesy of Dr. J. L. Hourigan, Senior Staff Officer, Sheep and Goat Diseases and Ectoparasites, Animal Disease Eradication Division, U.S. Department of Agriculture.

Goat and sheep brains were shipped to us as frozen whole tissue. Suspensions of tissue were prepared in 0.85 percent solution of sodium chloride without addition of serum or other proteinaceous materials; subsequently, on serial transmission of the scrapie agent from mouse to mouse we employed 0.2 M phosphate buffered saline (pH 7.4) containing a final concentration of 10 percent normal inactivated calf or rabbit serum and 100 units of penicillin with 100 micrograms of streptomycin per ml. Routinely male and female Swiss mice of the National Institutes of Health General Purpose stock were employed for these studies. In general, the amount of inocula was dependent upon the age of the test animal, e.g., newborn mice received 0.02 ml. via the intracerebral route or 0.05 ml. via the intraperitoneal route; weanling and adult mice were injected with 0.03 ml. intracerebrally or 0.1 ml. intraperitoneally. Brains of the affected mice in the goat brain and sheep brain passage series were harvested 4 to 15 months after inoculation and used immediately after harvest. Stock seed virus pools were stored in small aliquots in sealed glass ampules at -70°C . or at -20°C . following lyophilization. Infectivity end-point titrations were calculated by the method of Reed and Muench (11).

The sera employed in serological tests were obtained from sheep with naturally acquired scrapie and mice with experimentally induced scrapie-like disease; from mice, rabbits, and roosters following multiple

injections of mouse brain suspension known to contain the scrapie agent; and, for use as controls, from normal sheep, mice, rabbits, and roosters with histories of no known contact with scrapie-affected animals.

Serologic Tests.—Virus for neutralization tests was contained in buffered saline suspensions of affected mouse brains. Test sera were used undiluted following heat inactivation (sheep, 56°C ., 30 min.; rabbit, 62°C ., 30 min.; mouse, 60°C ., 20 min.; roosters, 60°C ., 20 min.). Serum-virus mixtures, except where noted in the results, were incubated at 37°C . for 1 hour and then inoculated into mice by the intracerebral route.

Complement-fixation tests were performed in the microtiter of Takatsy (12), as modified by Sever (13), employing two full units of complement and overnight primary incubation at 4°C . Antigens used were the supernatant fluids of 10 percent or 20 percent mouse brain tissue suspensions following low (2,500 r.p.m./30 min.) and high (13,000 r.p.m./30 min.) centrifugation, and mouse brain antigens prepared by the sucrose-acetone extraction method of Clarke and Casals (14).

Fluorescent antibody tests were performed using the direct and indirect techniques. Brains of moribund sheep and mice served as sources of antigen. In the indirect tests anti-sheep and anti-mouse sera prepared in rabbits and conjugated with isothiocyanate were used. In the direct test the serum of moribund mice tagged with isothiocyanate was employed.

Precipitin Tests.—The micromodification of the Ouchterlony double diffusion method in two dimensions described by Clarke (15, 16) employing 1 percent agar in 0.05 M NaCl-0.05 M borate, pH 9.0, was used in all studies.

RESULTS

Physical Properties of the Scrapie Agent, Compton Strain

Thermostability.—In an earlier communication (9) we presented data confirming Chandler's (5) observation on the resistance of the scrapie agent to heat. Because this characteristic contributes to the unorthodox nature of the scrapie agent when compared to more conventional viruses, additional experiments designed to determine more precisely the thermostability of the agent were performed. Suspensions of scrapie-infected mouse brain tissue were prepared in isotonic phosphate buffered saline (PBS) (pH 7.4), clarified by centrifugation at 2,500 r.p.m./30 min., and aliquots, both undiluted (10 percent w/v) and diluted 10-fold

TABLE I.—Thermostability of Scrapie Agent (Compton Strain) Following Exposure to 100° C. Temperature for Varying Periods of Time

Dilution of mouse brain suspension	Treatment										
	Experiment I			Experiment II							
	Exposure to 100° C./ 15 mins.			Serial dilutions exposed to 100°C. (time in minutes)							
	None	Diluted before exposure	Diluted after exposure*	None	2	5	10	15	30	45	60
10 ⁻¹	10/10**	5/8	4/5	5/6		0/3	0/3	0/5	0/4	0/4	0/7
10 ⁻²	10/10	3/6	1/5	6/6	2/4	0/6	0/3	0/2	0/4	0/4	0/4
10 ⁻³	10/10	0/7	1/8	6/6	0/5	0/3	3/7	0/5	0/5	0/5	0/5
10 ⁻⁴	3/3	0/10	1/6	3/3	0/5	0/3	0/4	0/6	0/6	0/5	0/4
10 ⁻⁵	7/8	0/5	0/6	2/3	0/4	0/7	0/4	0/6	0/5	0/4	0/4
10 ⁻⁶	0/9	0/8	0/6	0/5	1/5	0/2	0/2	0/6	0/5	0/5	
10 ⁻⁷	0/10	0/8	0/8	0/7	0/7	0/3					
10 ⁻⁸	0/10	0/8	0/8	0/4	1/3	0/3					
LD ₅₀ †	10 ^{-5.4}	10 ^{-1.6}	10 ^{-1.6}	10 ^{-5.0}	10 ^{-2.0}	<10 ^{-1.0}	~10 ^{-2.0}	<10 ^{-1.0}	<10 ^{-1.0}	<10 ^{-1.0}	<10 ^{-1.0}

*Exposed as a 10⁻¹ dilution.

**Mortality ratio—number of mice developing scrapie/number of mice inoculated; Experiment I held for 750 days post inoculation; Experiment II summarized 560 days post inoculation.

‡Mouse ICLD₅₀ endpoints computed by the method of Reed and Muench.

in PBS, were distributed into glass ampules which were then sealed and immersed in a stationary position in mechanically agitated constant temperature (100° C.) boiling water for varying exposure times. At predetermined intervals, samples were removed, placed in an ice bath, and in the case of the undiluted material serial 10-fold dilutions were prepared; all samples were then inoculated intracerebrally into litters of 3- to 5-day-old mice. The results of two experiments are summarized in table I. The findings in the first experiment show that while approximately 4 logs (mouse IC LD₅₀) of the scrapie agent, representing approximately 99 percent of the infectivity for mice, were destroyed by exposure to boiling water for 15 minutes, the activity of some of the infectious particles withstood the treatment. The results of the second experiment further indicate that, while the agent is not entirely resistant to heat inactivation at 100° C., exposure to such a temperature cannot be relied upon to produce total inactivation of its infectivity. In preparations containing less proteinaceous material the infectivity of the agent might be less heat resistant, although we have not yet demonstrated this. These results are in agreement with those of Eklund et al. (7) who emphasized the necessity of defining critically the methods used to test the heat stability of scrapie. Hunter (17) has shown the critical temperature of rapid heat inactivation of this agent to be 87.5° C.; at lower temperatures inactivation is very slow.

He thus suggests that the bulk of the agent exists in a heat-stable form that probably contains double-stranded DNA.

Effect of Diethyl Ether on Infectivity.—Stock seed suspensions of scrapie-infected mouse brain were diluted in phosphate buffered saline and treated with diethyl ether in the manner described by Theiler (18). Following incubation at 4° C. for 16 to 18 hours treated and untreated dilutions were inoculated intracerebrally into weanling mice (8 to 10 grams). The results of these tests show that the infectivity titer of the scrapie agent was reduced 1.7 to 2.2 logs following exposure to ether, thereby suggesting that the agent is sensitive to this organic compound although less so than are the arboviruses (see table II).

TABLE II.—Effect of Diethyl Ether on Scrapie Agent

Dilution of inoculum	Treatment		
	None	Incubation (4° C. 16–18 hrs.)	Exposure to diethyl ether (4° C. 16–18 hrs.)
10 ⁻¹	7/7	5/5	5/5
10 ⁻²	6/6	5/5	5/5
10 ⁻³	6/6	5/5	5/5
10 ⁻⁴	6/6	6/6	3/3
10 ⁻⁵	6/6	6/6	4/5
10 ⁻⁶	6/6	5/5	2/5
10 ⁻⁷	5/7	4/5	1/5
10 ⁻⁸	2/7	3/5	0/5
LD ₅₀	≥10 ^{-7.5}	≥10 ^{-8.0}	10 ^{-5.8}

TABLE III.—Determination of Particle Size of Scrapie Agent by Gradacol Membrane Filtration

Dilution of inoculum ¹	Pre-filtration infectivity titer	Ability of selected filtrates to induce scrapie disease in mice					
		520 m μ filtrate	220 m μ filtrate	82 m μ filtrate	43 m μ filtrate	27 m μ filtrate	8 m μ filtrate
10 ⁻¹					6/6 (126-195)	0/10	0/10
10 ⁻²				6/6 (116-164)	9/9 (143-192)	0/8	
10 ⁻³	9/9 (129-197) ²	6/6 (130-198)	6/6 (141-191)	6/6 (143-171)	5/6 (161-192)		
10 ⁻⁴	11/11 (144-208)	6/6 (143-220)	6/7 (140-188)				
10 ⁻⁵	5/5 (127-208)	2/3 (146-200)	0/2				
10 ⁻⁶	0/7	4/9 (183-201)	0/6				
10 ⁻⁷	0/6	0/6					
10 ⁻⁸	0/2	0/7					
10 ⁻⁹		0/11					
LD ₅₀	10 ^{-5.5}	10 ^{-5.7}	10 ^{-4.4}	>10 ^{-3.0}	>10 ^{-3.0}	<10 ^{-1.0}	<10 ^{-1.0}

¹ Ten percent mouse brain suspension in phosphate buffered saline (pH 7.4) clarified by centrifugation at 5000 rpm/30 minutes.

² Number of mice developing scrapie/number of mice inoculated followed by the range in days post inoculation of onset and death for a given group of mice.

Estimate of Size by Filtration.—A 10-percent suspension of scrapie-infected mouse brain tissue was centrifuged at 5,000 r.p.m. for 30 minutes. The supernatant fluid was serially passed through a gradacol membrane of 520 m μ pore size, a millipore membrane of 220 m μ and then successively through gradacol membranes of the following pore size: 82 m μ , 43 m μ , 27 m μ , and 8 m μ . After each filtration step aliquots in several dilutions were inoculated intracerebrally into litters of 3- to 5-day-old Swiss mice. As shown in table III deaths attributable to the scrapie agent occurred in all filtrates down to and including 43 m μ , but infectious particles did not pass the 27 m μ membrane. After more than 1 year of observation mice inoculated with the 27 m μ and 8 m μ filtrates have remained healthy. These data demonstrate that the scrapie agent is of a small size. Application of Black's constant (0.64), as a means of expressing the relationship between the size of the infectious particle and the limiting pore diameter through which the particle passed, places the size of the scrapie infectious particle in the range of 17.3 m μ to 27.5 m μ (19). This confirms the early observation of Stamp et al. (20) that the sheep scrapie agent failed to pass through a 27 m μ gradacol membrane.

Biological Properties of the Scrapie Agent, Compton Strain

Host Susceptibility.—During the course of our studies on the physical and biological properties of the Compton strain of scrapie agent we have observed no significant difference in the susceptibility to infection between male and female mice (see table IV). On

primary passage of this strain of the scrapie agent results suggested that although the disease could be induced in mice by the intracerebral and the intraperitoneal routes of inoculation, the incubation period was longer and the infectivity titer lower following peripheral inoculation. However, as shown in table V, on subsequent serial mouse to mouse passage, providing additional adaptation of the agent to this host, no significant difference in infectivity titers was observed though the incubation continued to be longer following intraperitoneal inoculations.

Serological Tests.—No differences in neutralizing capacity between sera obtained from normal and affected animals, nor between preinoculation and post-inoculation sera from "immunized" animals were

TABLE IV.—Results of Inoculation of Goat-Passed Scrapie Agent Into Male and Female Mice

Dilution of infected mouse brain suspension ¹	Number of mice developing scrapie/number mice inoculated ²	
	Male	Female
10 ⁻¹	10/10	9/9
10 ⁻²	9/9	10/10
10 ⁻³	3/3	7/7
10 ⁻⁴	1/1	3/3
10 ⁻⁵		2/2
10 ⁻⁶	2/2	2/2
10 ⁻⁷	0/6	0/7
10 ⁻⁸	2/2	0/6
LD ₅₀	10 ^{-6.7}	10 ^{-6.5}

¹ Inoculated into 6-8 gm. Swiss-Webster mice via the IC route.

² Computed at 712 days post inoculation.

ABLE V.—Relative Susceptibility of Mice to the Scrapie Agent (Compton Strain) Inoculated Intracerebrally or Intraperitoneally

Inocula	Passage level	Route of inoculation	Range of clinical illness (onset-death days post inoculation)	Infectivity titer LD ₅₀ *
Goat brain 9th passage	M/1	IC	216-491	10 ^{-5.4}
		IP	252-568	10 ^{-2.8}
Mouse brain, M/1	M/2	IC	123-258	10 ^{-6.5}
		IP	182-412	10 ^{-6.3}
Mouse brain, M/2	M/3	IC	153-248	10 ^{-5.7}
		IP	182-258	10 ^{-5.2}
Mouse brain, M/3	M/4	IC	146-248	10 ^{-5.3}
		IP	178-260	10 ^{-5.1}

*Titration were performed in young weanling mice (6-8 grams in size) of the Swiss-Webster strain, NIH general purpose stock. Infectivity titers were computed by the method of Reed and Muench.

obtained. Furthermore, none of the sera produced significant neutralization of the seed virus. Results were also negative in complement-fixation, precipitation, and in the direct and indirect fluorescent antibody tests with the same reagents.

Data representative of our attempts to demonstrate neutralization of the infectivity of the agent inducing scrapie-like illness in mice by sera obtained from scrapie-infected sheep and mice, and mice, rabbits, and roosters that had received multiple injections of mouse

brain suspension known to contain the scrapie agent are illustrated in table VI.

Failure to Demonstrate Immune Tolerance in Test Mice.—An experiment was carried out in an attempt to determine whether or not immune tolerance could be demonstrated in mice injected early in life with mouse brain infected with the Compton strain of scrapie. This approach was taken because it had been shown by Hotchin (21) that neonatal inoculation of mice with lymphocytic choriomeningitis virus induces a persistent tolerant infection in which high virus titers persist for many months in the absence of clinical signs of disease.

The results of the experiment, given in table VII, show that no such tolerance was demonstrated in mice injected with the scrapie agent. Mice injected 12 to 72 hours after birth were as susceptible to fatal scrapie-

TABLE VII.—Mortality and Mean Incubation Periods in Mice of Different Ages Following Intracerebral Injection of 10⁴ Mouse ICLD₅₀ of "Scrapie" Agent

Age of mice at time of inoculation (days)	Number mice in group	Number mice developing fatal "scrapie"	Percent mortality	Mean incubation period (days)
1/2	46	44	96	148
3	26	25	96	150
5	39	39	100	150
9	31	28	91	150
60	18	18	100	149

TABLE VI.—Attempts to Demonstrate Neutralizing Antibody to Mouse Scrapie Agent, Compton Strain SPG9

Scrapie agent mixed with: ¹										
Virus dilution	Sheep serum ^a		Mouse serum ^a		Mouse immune ascitic fluid ^b		Rooster serum ^b		Rabbit serum ^b	
	Normal	Scrapie	Normal	Scrapie	Normal	Scrapie	Normal	Scrapie	Normal	Scrapie
10 ⁻¹	6/6 ²	8/8	10/10	8/8	5/5	5/5	5/5	5/5	5/5	5/5
10 ⁻²	8/8	8/8	10/10	8/8	5/5	5/5	5/5	5/5	5/5	5/5
10 ⁻³	7/7	8/8	10/10	8/8	5/5	5/5	5/5	5/5	5/5	5/5
10 ⁻⁴	8/8	8/8	3/3	4/4	5/5	5/5	5/5	5/5	5/5	5/5
10 ⁻⁵	8/8	7/8	7/8	6/7	5/5	5/5	5/5	5/5	5/5	5/5
10 ⁻⁶	2/9	0/5	0/9	0/7	5/5	5/5	5/5	5/5	5/5	5/5
10 ⁻⁷	0/9	0/8	0/10	0/5	5/5	5/5	5/5	5/5	5/5	5/5
10 ⁻⁸	0/9	0/8	0/10							
MICLD ₅₀	10 ^{-5.6}	10 ^{-5.4}	10 ^{-5.4}	10 ^{-5.4}	≥10 ^{-7.5}	≥10 ^{-7.5}	≥10 ^{-7.5}	≥10 ^{-7.5}	≥10 ^{-7.5}	≥10 ^{-7.5}

¹ Mixture of undiluted serum, or ascitic fluid (inactivated 56° C./30 min.) and 10 fold dilutions of mouse brain infected with scrapie virus:

^a Serum-virus mixtures incubated at 37° C. for 1 hour, then inoculated into 6-8 gm. mice by the intracerebral route.

^b Serum-virus mixtures incubated at room temperature (25°-27° C.) for 16-18 hours, then inoculated into litters of 5- to 7-day-old mice by the intracerebral route.

² Number of mice developing scrapie/number of mice inoculated.

TABLE VIII.—Effect of Mouse Scrapie Agent on Pregnant Mice and Their Progeny

Virus strain	Virus dilution	Injected days predelivery	Number of animals developing scrapie ¹		
			Inoculated mothers	Own progeny	Foster progeny
Compton SPG9:M4	10 ⁻¹	4	0/1	0/10 (300) ²	0
	10 ⁻²	4	2/2 (193–225) ³	8/15 (212–245)	eaten
	10 ⁻³	4	2/2 (194–225)	eaten	eaten
	10 ⁻⁴	7	2/2 (281–299)	3/3 (225–236)	0/5
	10 ⁻⁵	8	2/2 (200–244)	eaten	0/10 ⁴

¹Own progeny removed immediately following birth and placed with clean uninoculated lactating females; foster progeny from clean mothers placed with inoculated lactating females.

²Indicates that no deaths have occurred during the 300 days the experiment has been under observation.

³Mortality ratio followed by the number of days post inoculation (or delivery) of onset and death.

⁴4/10 mice in this group developed symptoms suggestive of scrapie illness on day 218 but all mice had recovered by day 244.

like infections as were older mice injected with aliquots of the same material. Further, the age of the mice at the time of inoculation did not affect the mean incubation period of the scrapie-like disease. Thus, we were unable to obtain support for the hypothesis that the prolonged incubation period that characterizes the scrapie-like illness in mice might have as its underlying cause a persistent tolerant state. These data are in agreement with those of Stamp et al., who showed that Cheviot lambs inoculated intracerebrally within 24 hours of birth developed scrapie before they were 9 months old.

It was of interest to determine whether or not scrapie disease would develop in mice born of pregnant females inoculated during the gestation period but separated from their own dams at delivery. The data in table VIII clearly show that mice do become infected prenatally or perinatally, develop clinically recognizable disease and die following an incubation period not significantly different from that observed as occurring in their inoculated mothers. Thus, one might conclude from this experiment that the disease observed by Eklund et al. (7) in litters of suckling mice born to females inoculated intracerebrally were infected before or during birth, rather than by post-delivery contact. However, there is some previous evidence suggesting contact infection between mice, and a preliminary report of our experience with such infection will be presented by Dr. J. A. Morris (22).

Pathogenicity of Scrapie-Infected Sheep Tissues for Mice.—Since our first paper describing encephalopathy in mice following inoculation of central nervous tissue from a sheep with naturally occurring scrapie infection we have received a number of tissue specimens from sheep in the United States whose deaths were confirmed on clinical and neuropathological

grounds by the U.S. Department of Agriculture as due to scrapie. While it has not been possible to attempt isolation studies on all specimens submitted we have been able to induce encephalopathy in groups of mice inoculated with tissues from nine separate cases. Results obtained with seven of these cases, including the development of scrapie-like illness in mice inoculated with the serum of an infected ram (strain 001388), are summarized in table IX. To date, because of space limitations, we have been successful in serially passing only three of these strains in Swiss mice in the laboratory.

TABLE IX.—Pathogenicity of Scrapie-Infected Sheep Tissues for Mice ¹

Strain designation	Inocula	Scrapie	Range of illness (days)	
		positive/inoculated ²	onset	death
001388	Serum	4/10 (40%)	360	404
001389	CNS	4/49 (8%)	490	513
001400	CNS	3/40 (7%)	101	250
001401	CNS	4/40 (10%)	297	476
001402	CNS	18/40 (45%)	161	466
001403	CNS	5/40 (12%)	165	467
001404	CNS	8/40 (20%)	465	523

¹Cases diagnosed by U.S. Department of Agriculture Field Veterinarians; specimens submitted to the NIH for study through the courtesy of Staff Veterinarians of the USDA.

²Figures adjusted and do not include animals that died during the course of the experiment due to nonspecific reasons.

It is of importance to note that the original and passage materials in these studies were free of detectable pleuropneumonia-like organisms (PPLO), *Listeria monocytogenes*, Tyzzer's agent and of bacteria capable of growth on blood agar and in thioglycollate

broth. Histopathological study of tissues of mice in this sheep passage series were carried out in collaboration with Mrs. Elisabeth Beck, who demonstrated that the lesions in the brains and spinal cords of the affected mice were similar to those described by Pattison and Smith (23) as being typical of scrapie.

DISCUSSION

The data presented in this paper provide additional characterization on the nature of experimentally induced encephalopathy in mice inoculated with the Compton strain of goat scrapie and serum and brain tissue suspension from sheep with naturally acquired scrapie in the United States. At this time no information has been obtained that would suggest possible variations between the English and American strains of the self-replicating agent, or virus, that causes the progressive degenerative disease of the nervous system of mice with an incubation period of 4 to 15 months. It should be noted that we have not proved that the illness developing in mice following injection of bacteria-free scrapie sheep and scrapie goat brain is the etiologic agent of scrapie. The test animals develop clinical and pathological findings which are similar to the manifestation of scrapie in sheep and goats. Clinically they develop hyperexcitability, drowsiness, waddling incoordinated gait, emaciation, stiff almost cataleptic tail and, in many instances, a urinary and fecal incontinence and persistent priapism; and the neurohistological lesions consist of marked astrogliosis, vacuolation, and loss of neurons and myelin degeneration predominantly of the cerebellar and hypothalamic systems, and absence of inflammatory lesions.

The negative results we have obtained in our serological studies on attempts to demonstrate antibody against the scrapie agent in the serum of sheep, goats, mice, rabbits, and roosters may be due to one or a combination of factors. Many possible explanations require investigation: (1) the agent is infective nucleic acid without a protein envelope and thus does not stimulate antibody formation; (2) the test sera were devoid of antibody in levels high enough to be detectable by the techniques employed; (3) test antigens were antigenically deficient; and (4) improper testing procedures were employed. Pursuant to the latter suggestion we are modifying the existent procedures of neutralization tests, and complement-fixation, precipitin and fluorescent antibody techniques. Plescia, Braun, and Palczuk (24), have reported a method of producing antibodies to purified nucleic acids which we shall use with the scrapie agent.

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Observations on the Pathology of Experimentally Produced Scrapie

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We have been investigating some of the mice inoculated with scrapie which were sent to us by Dr. Gajdusek and Dr. Gibbs. This is a preliminary report on our findings.

The brains were fixed *in situ* and removed after fixation. Histological sections were cut serially throughout the whole cerebrum, cerebellum and brainstem. Representative levels of the spinal cord were examined. The staining methods employed were the same as those used in our investigation of scrapie in sheep.

In common with all previous investigators we found widespread “nonspecific” changes, such as striking astrocytic hypertrophy, marked status spongiosus and various types of neuronal degeneration. In addition, however, there was degeneration of specific neuronal systems, in fact of those systems which were also affected in the natural disease of sheep, i.e., the cerebellar and the hypothalamo-neurohypophysial system.

In the cerebellum there was marked loss of Purkinje cells and rarefaction of the granular layer (fig. 1 B). Empty baskets were present. There was some degree of nerve fibre degeneration and occasional torpedolike swellings were seen (fig. 1 C). The Bergmann glia was markedly proliferated in some cases. PAS-positive, doubly refractile plaques were found in the cere-

bellar cortex of some cases. No significant changes were seen in the cerebellar white matter.

In the motor system the Vth, VIth, VIIth and XIIth nerve nuclei showed typical vacuolated neurones and these were particularly numerous in the VIIth nucleus (fig. 2). In the spinal cord there was some loss of anterior horn cells as well as vacuolation.

The hypothalamo-neurohypophysial system showed marked degeneration with considerable neuronal loss in the supraoptic and paraventricular nuclei (fig. 3 A–D) and some excess of neurosecretory material in the median eminence.

As in natural scrapie the disease process caused a specific degeneration in both the cerebellum and the hypothalamus of inoculated mice. In addition, however, the motor nuclei including the anterior horns were involved. Whilst we did not observe a degeneration of the motor system in our sheep with natural scrapie such changes have been reported by others. It thus seems that the agent, apart from causing widespread “nonspecific” changes in the central nervous system, also attacks the same specific systems as are damaged in the natural disease. This is true not only for mice affected by experimental scrapie but also for inoculated sheep and goats of which we have examined several specimens.

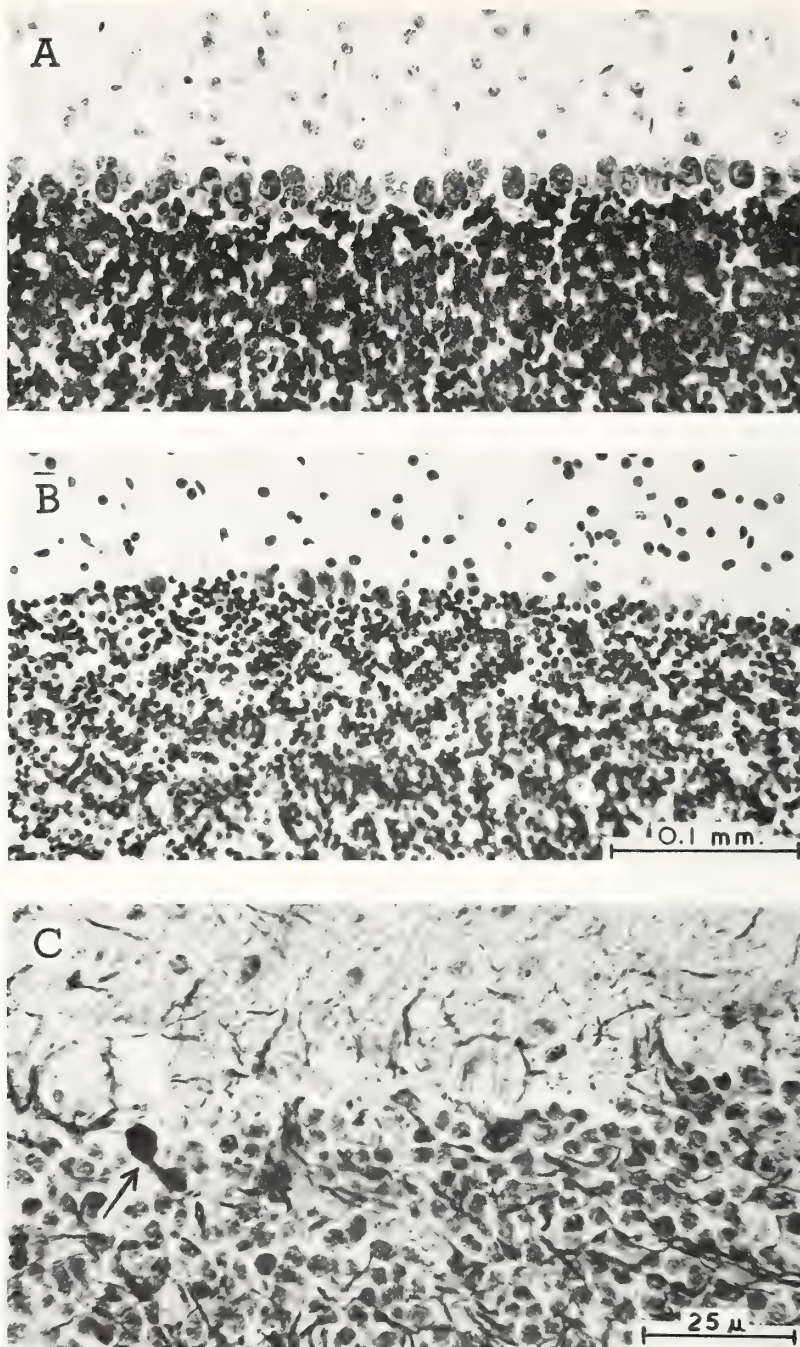


FIGURE 1.—(A) Cerebellar cortex of normal mouse. Note regular arrangement of Purkinje cells. (B) Scrapie mouse. Considerable loss of Purkinje and granule cells. (C) Scrapie mouse. Note torpedolike swelling on axon of degenerating Purkinje cell (arrow) and also empty baskets. (A, B Nissl stain; C Glees-Marsland silver impregnation).

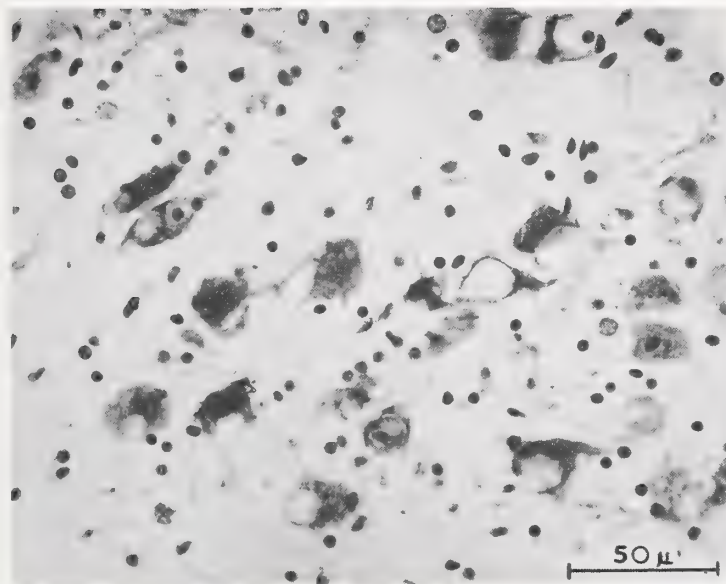


FIGURE 2.—Nucleus of VIIth nerve showing numbers of vacuolated motor neurones. (Nissl stain.)

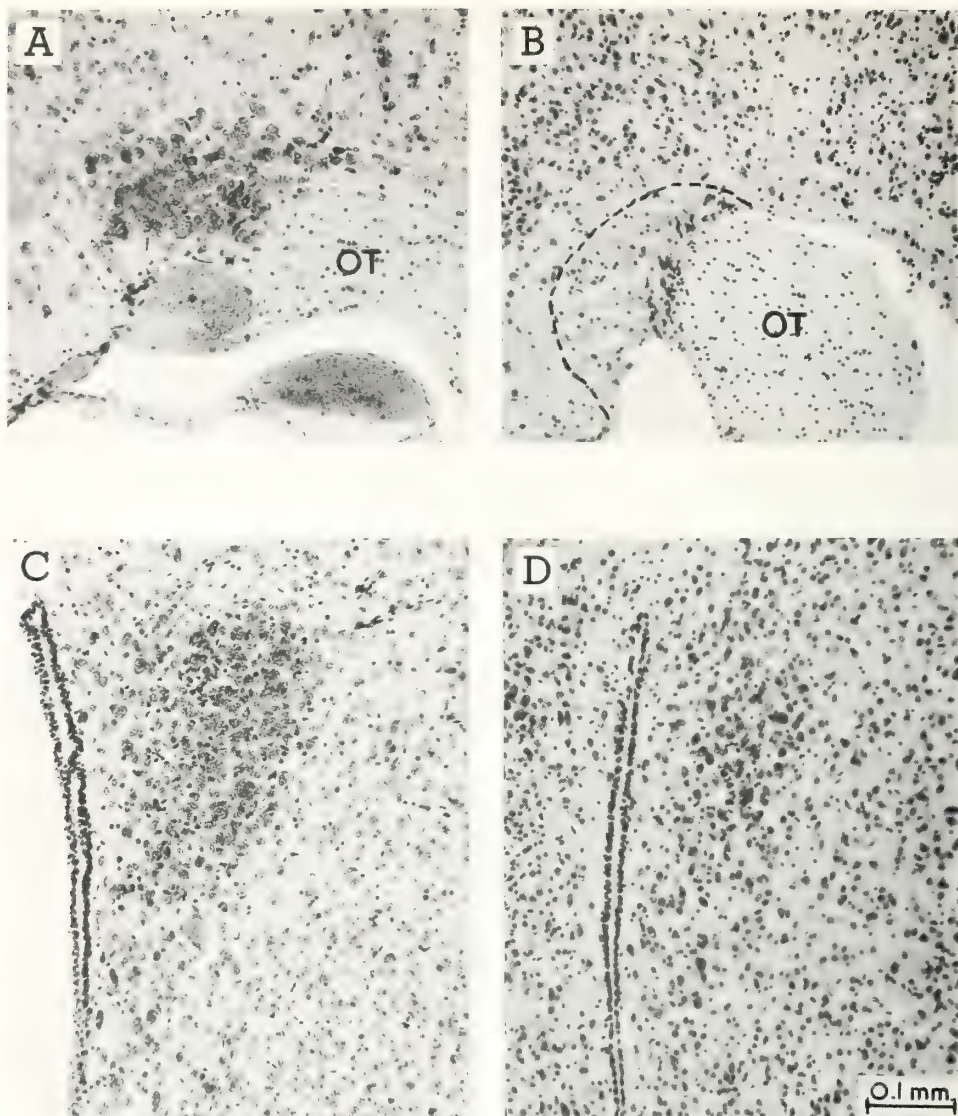


FIGURE 3.—(A) Supraoptic nucleus of normal mouse. (B) Supraoptic nucleus of scrapie mouse. Note almost complete loss of neurones. (The territory of the nucleus has been outlined. OT—optic tract.) (C) Paraventricular nucleus of normal mouse. (D) Paraventricular nucleus of scrapie mouse. Note severe reduction in the number of neurones. (Nissl stain.)

Pathogenesis of Scrapie Virus Infection in the Mouse

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Although scrapie has been studied for many years, its pathogenesis is still poorly understood (1). This status has prevailed largely because of the lack of methods for adequately characterizing the underlying infectious process. Now, however, with the adaptation of scrapie virus to the mouse (2), a convenient biologic system is available to do so. Accordingly, we have used this experimental model to obtain quantitative data on the growth and temporal distribution of the virus in relation to onset of disease in this host. Because this study will be reported in full elsewhere, only a summary account is given here.

MATERIALS AND METHODS

Weaned Swiss mice were inoculated subcutaneously with $10^{5.5}$ mouse LD_{50} of scrapie virus. At intervals thereafter, they were killed in groups of six. Three mice were examined histologically. For the detection

of virus, serial 10-fold dilutions through 10^{-8} were made from pooled specimens of the other three mice; .03 ml. of each dilution was injected intracerebrally into six weaned mice, which were observed for periods up to 1 year. The occurrence of clinical scrapie in these mice was taken as evidence of virus in the inoculum (3).

RESULTS

As indicated in table I, virus appeared first in the spleen and peripheral lymph nodes (axillary and inguinal), where it persisted in large amounts throughout the prolonged course of the infection. Soon after its appearance here, virus was found in the thymus and submaxillary salivary glands. Then, after slow replication in the salivary gland and lymphocytic tissues, virus spread to other structures, such as the lungs and intestine (lower ileum and upper colon), where it re-

TABLE I.—Growth and Temporal Distribution of Scrapie Virus in Swiss Mice Inoculated Subcutaneously

Organ ¹	Weeks after inoculation											
	1	2	4	8	12	16	20	24	28	28 ²	32 ²	36 ²
Spleen	$3 \cdot 10^{-4}$		10^{-3}	10^{-6}	10^{-6}	10^{-7}	10^{-7}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-4}
Peripheral lymph nodes			10^{-3}	10^{-6}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-5}
Submaxillary salivary gland				10^{-6}	10^{-5}	10^{-6}	10^{-6}	10^{-6}	10^{-7}	10^{-7}	10^{-5}	10^{-3}
Thymus				10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-1}	10^{-5}	10^{-5}
Lung					10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-2}	10^{-2}	10^{-1}	
Intestine					10^{-3}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-3}	10^{-5}
Spinal cord					10^{-1}	10^{-5}	10^{-5}	10^{-7}	10^{-6}	10^{-7}	10^{-8}	10^{-6}
Brain						10^{-4}	10^{-3}	10^{-6}	10^{-7}	10^{-7}	10^{-6}	10^{-6}
Bone marrow (femur)								10^{-1}	10^{-3}	10^{-4}	10^{-2}	10^{-5}
Liver									10^{-1}	10^{-1}	10^{-1}	
Uterus							10^{-1}			10^{-2}		

¹ Pooled from 3 mice and each dilution inoculated intracerebrally in 6 mice.

² Mice affected with clinical scrapie.

³ Highest dilution in which virus was detected. Blanks indicate virus was not detected.

mained in relatively small amounts. Virus did not appear in the central nervous system until late in the long incubation period. It was found first, about 3 months after inoculation, in the spinal cord and then about 1 month later in the brain. From then on, the amount of virus in the central nervous system gradually increased until clinical signs of scrapie began to appear 5 to 6 months after inoculation. Virus was still widely distributed when the mice died, but at no time was it detected in the blood clot, serum, or kidneys.

Histologic changes, mainly astrogliosis, occurred in the central nervous system at about the time of onset of clinical signs. Appearing first in the spinal cord, the astrogliotic response was always much more conspicuous than overt changes in nerve cells.

DISCUSSION

The slowness of replication and spread of scrapie virus in the mouse contrasts sharply with the course of events occurring in mice inoculated with viruses causing acute infection. Thus, in scrapie virus infection the events are measured in weeks and months rather than in hours and days. Moreover, the slow, steady increase in the amount of scrapie virus in the mouse occurs in the absence of any evident defense response on the part of the host. The early predilection of the virus for structures important in the defense mechanism of the body, i.e., those containing abundant lymphocytic tissue, could have a bearing on the relentless progression of the disease. For example, the continued replication of virus and its eventual spread to the central nervous system, where destructive changes lead to death of the host, may occur because the formation of protective antibody is inhibited. On the other

hand, perhaps the slow replication of virus in lymphocytic tissues results in a state of tolerance in which the usual defense mechanisms are not set in motion.

Among other factors that may help explain the pathogenesis of scrapie is the nature of the virus itself. The unusual resistance of the virus to heat and to formalin suggests that its structure differs in some fundamental way from that of the relatively labile viruses causing acute disease. Possibly, the structure is such that scrapie virus does not stimulate the immune mechanisms of the body.

Whether the pattern of virus growth observed in the mouse is the same in the sheep and the goat is not known. Nevertheless, the meager data available on the distribution of virus in these animals suggest a similar course of events.

In the mouse, the astrogliotic response appears early and throughout the course of the disease it is generally more conspicuous than the neuronal alterations. This suggests that the astrogliosis may be the primary and most significant cellular response in scrapie. Perhaps, then, the dysfunction of nerve cells responsible for the severe neurologic disturbances characterizing this disease occurs indirectly as a consequence of virus-induced antecedent changes in astrocytes.

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Tissue Culture Studies on Scrapie

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INTRODUCTION

In the initial approach to the problem of trying to grow scrapie agent in tissue culture the following methods were adopted:

(a) Outgrowth of tissues from animals affected at various stages were compared with similar cultures of normal animals; a wide range of tissues from sheep and goats were examined.

(b) Normal adult and foetal sheep and goat tissues were used as substrates and inoculated with scrapie material.

(c) Line cells were obtained from other laboratories and also developed from foetal tissues. These were then examined for abnormal growth after contact with the scrapie agent.

(d) Attempts were made to demonstrate inhibition of cell metabolism by the scrapie agent and also to demonstrate the interference phenomenon with other viruses.

These studies have given no evidence of cytopathogenicity or any consistent departure from the normal even when cultures were observed for periods up to 7 weeks. The investigations were made with the scrapie agent of the Cheviot line and of a goat-adapted line, both being available as biologically-tested pool materials. Both were derived from the same source, namely, the Wilson strain of Cheviot agent which originated at Moredun. At the time it was only possible to test material for infectivity by using goats or sheep and although some tissue culture series were tested it was not practicable to do this routinely or extensively.

When the scrapie agent was passed in mice (Chandler (1), 1961) these animals became freely available

both as donors and indicators of the agent so that more extensive testing of tissue culture systems became possible.

Mouse-Adapted Scrapie Agent in Tissue Culture

1. Mouse Spleen Plasma-Clot Cultures.—Spleen fragment cultures from mice infected with a third-mouse-passage of goat scrapie (drowsy type) were initiated from mice showing signs typical of mouse scrapie. These were maintained with regular changes of medium and aliquots of supernatant fluid after disruption of the cells were harvested at intervals and injected into mice. It was shown that the agent survived for at least 16 days in cultures of this type. In another experiment one of 10 mice developed scrapie lesions following intracranial inoculation with the harvest from a 28-day spleen culture.

2. Primary Embryo Mouse Tissue Culture as Substrate.—These cultures are of mixed cells but fibroblasts predominate and they can be maintained for up to 2 months. Primary and secondary cultures of mouse scrapie were carried out for varying periods of time, the material being tested in mice for infectivity. The data so far obtained is as follows:

(a) With cell-free extracts of infected mouse brain as inoculum, the agent survived up to 48 days in cultures incubated at 32° C. and at 37° C. The medium was changed every 4 to 5 days.

(b) The scrapie agent survived in subcultures of (a) made at 39 days, the secondary culture having been incubated for a further 19 days. Subsequent subcultures were negative.

(c) Spleen extracts from mice, whose brains were positive for scrapie, were cultured as for (a) and (b).

When tested in mice the cultures failed to produce scrapie in mice after 10½ months' incubation.

(d) Extracts of infected mouse brain and spleen have not produced a cytopathogenic effect (C.P.E.).

Blind Passage of Material from Cases of Scrapie

Many unsuccessful attempts to culture the scrapie agent have been made from tissues which originated as experimental passage material from Cheviot sheep, sheep scrapie adapted to goats, or Suffolk sheep and goat scrapie adapted to mice. However, a series of blind passages of brain tissue from a number of unusual cases of the disease have been carried out on primary pig kidney and cytopathogenic agents have been isolated.

SB 72

This was isolated from the brain of a 3-year-old half-breed sheep, which was one of three sent to the diagnostic laboratory in Edinburgh with symptoms of acute encephalitis simulating louping-ill. Neither a virus nor a bacterial agent was recovered on primary culture or by mouse inoculation. Brain sections examined some weeks later showed that all three sheep had lesions characteristic of scrapie. Further tissue cultures and mouse inoculations were subsequently made from brain stored at -20°C .

GB 7304

Tissues were cultured from a goat which had developed scrapie without inoculation. It had been in contact with goats inoculated with scrapie but may have been a rare natural case (Mackay and Smith, (2), 1961).

GB 8B13 and 8B15

These isolates were obtained from goats injected with Suffolk sheep-to-mouse strain, ME7, after two passages in mice followed by two passages in goats.

PRELIMINARY EXAMINATION OF CYTOPATHIC AGENTS

Microscopic Appearance

Each of the source materials caused a similar C.P.E. after three to five blind passages. Most subsequent examinations with the exception of the neutralisation tests have been carried out with SB 72. In the first instance it was noted that some cells were detached from the coverslips at 5 to 7 days but the remaining cells appeared healthy. After the fifth passage this effect was noted at 48 hours and at 3 days cytopathogenicity was usually complete. Examination of coverslips between 6 and 24 hours, as fresh preparations under phase contrast, showed cells that were grossly vacuolated and with bubble-like processes extending outwards from the cell wall (figs. 1 to 3). Occasionally refractile material was noted in the vacuoles or in

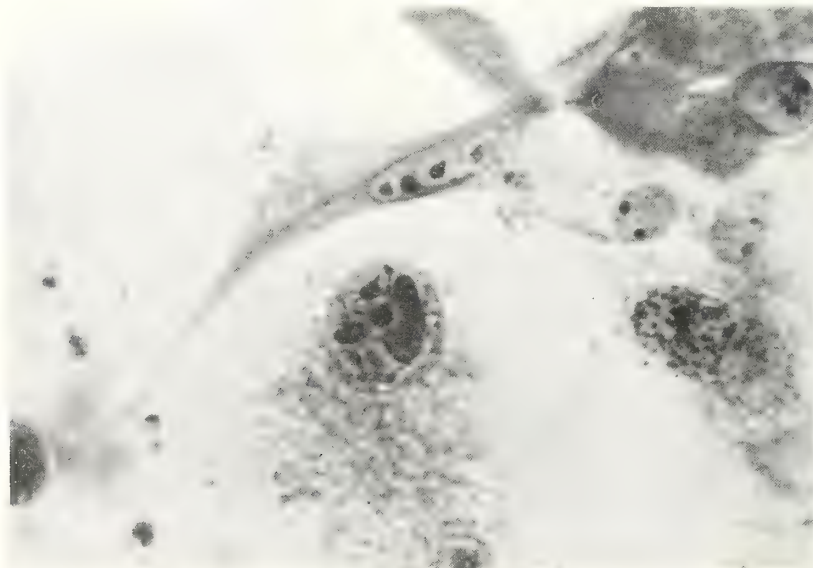


FIGURE 1 $\times 770$

FIGURES 1-4.—Photomicrographs of C.P.E. obtained with SB 72 in primary pig kidney.



FIGURE 2.—SB 72 in primary pig kidney $\times 880$.

the cell cytoplasm adjacent to the processes. The cell nucleus was often distorted and nucleoli displaced by the presence of the vacuoles (fig. 4). Stained by Mann or Acridine these now seem to be inclusion bodies. Studies on the cytopathogenicity have not been completed but the effects are remarkably similar to the morphological changes which cause loss of viability in Ehrlich ascites tumour cells after incubation with 0.0096 per cent solution of formaldehyde (3×10^{-3} M) (Ratzenhofer and Zangger (3), 1963).

Virus Titre

The agent reproduced to effect of titres of $10^8 > 10^7$ and this has been maintained serially for over 35 passages. Plaque formation can be demonstrated. There are about 10^8 p.f.u. per ml. of fluid cells in roux flasks harvested at 3 days.

Heat Resistance

Affected pig kidney cells in the presence of 10 per cent calf serum were heated at 80° C. for 20 minutes

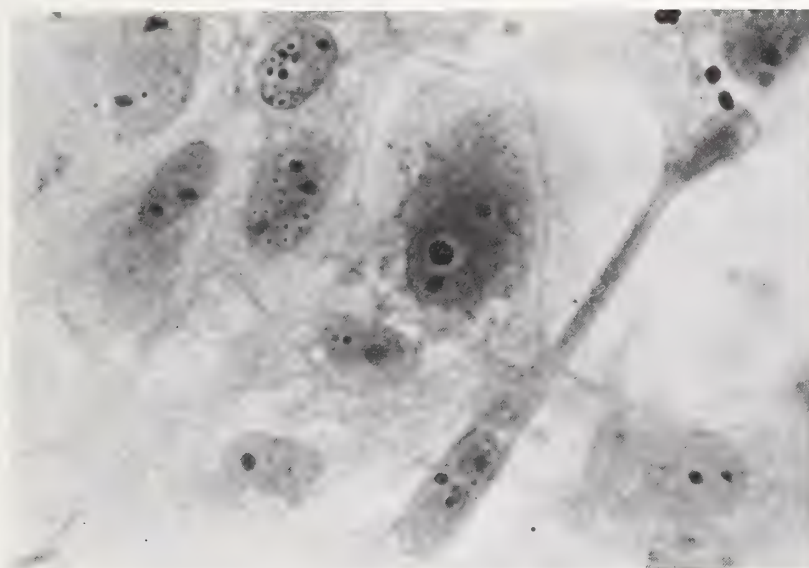


FIGURE 3.—SB 72 in primary pig kidney $\times 770$

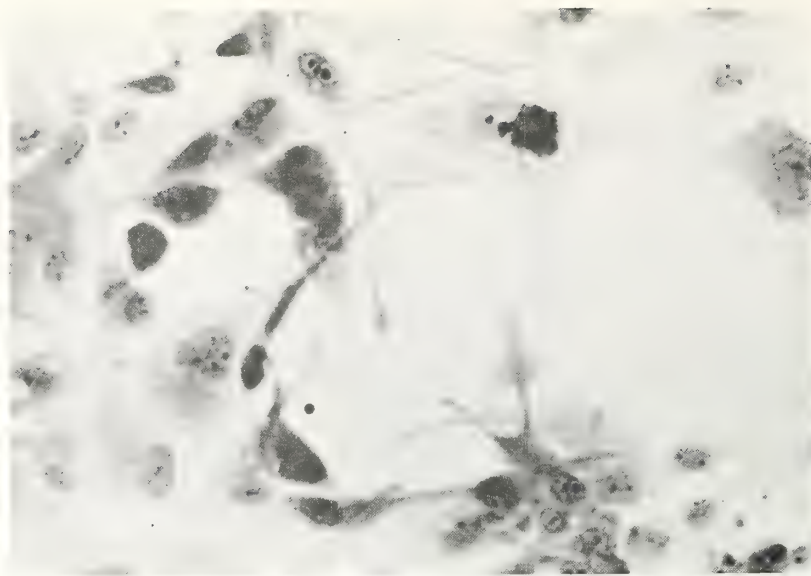


FIGURE 4. SB 72 in primary pig kidney $\times 220$.

and the titre was reduced from 10^{-7} to 10^{-2} per ml. of the cell-free supernate. After heating at 100°C . for 10 minutes the agent survived in one out of three trials. In serum-free medium the agent did not survive heating to 100°C .

Resistance to Ether

After treatment with 20 percent ether at 4°C . for 15 hours the agent could be passed in culture. No titration has been carried out.

Resistance to Freezing and Dying

The agent can be stored at -20°C . for at least 4 weeks and survives freeze-drying. No potency tests have been made.

Growth on Other Tissues

No evidence of cytopathogenicity has been found in embryo mouse tissue culture, mouse spleen macrophages, sheep kidney, calf kidney or BHK 21 inoculated with pig kidney tissue culture passages of SB 72. A pig line cell (STICE) will support growth of SB 72.

Neutralisation Tests

Antiserum against SB 72, prepared in rabbits, neutralised 100 TCD₅₀ of homologous agent to titre of 1:60. Cross-neutralisation tests indicated that all the agents had common antigens. Sera prepared from noninfected pig kidney cells did not neutralise the effect.

Antiserum prepared against T 80 and V 13 pig enteric viruses did not neutralise SB 72 but some neutralisation was obtained with antiserum to Talfan virus.

Electron Microscope

Studies with crude supernates of SB 72 have demonstrated hexagonal bodies of about $25\text{ m}\mu$ in size with subunits.

Passage in Mice

A 10 percent brain supernate of the original sheep brain SB 72 when injected intraperitoneally into sucking mice killed the mice in 18 days. This effect could not be repeated by further passage of brains or spleens of moribund mice but the same cytopathic agent as described above was recovered in tissue culture from spleen and brain. The 10 percent brain emulsion of SB 72 does not affect weaned mice adversely when injected intraperitoneally or intracranially.

Pathogenicity for Animals

The animal inoculation experiments summarized in table I are in progress but at present no signs of pathogenicity have been noted.

CURRENT WORK

Routine cultures are being carried out with tissues of all sheep and goats passing through the Department of Experimental Pathology, to correlate the isolation of agents with histopathology. To date 18 isolates of a similar nature to those described have been made. Concurrent serological studies are also in progress. Of one group of sheep injected with SB 72 agent, 23 of 31 have shown a rise in neutralising titre from 0-1:5 to 1:20 to 1:80. This group of sheep were injected

TABLE I

Agent	Passage	Time after injection			
		Mice	Sheep	Piglets	Goats
SB 72	10 percent sheep brain	Up to 12 months	Not done	Not done	Not done
SB 72	Tissue culture 2d pass	Up to 11 months	Up to 15 months	Not done	Not done
SB 72	Tissue culture 10th pass	Up to 12 months	Up to 15 months	Not done	Not done
SB 72	Tissue culture 20th pass	Up to 12 months	Not done	Up to 8 months	Not done
All isolates	Tissue cultures of 8th-20th pass + normal brain	Up to 6 months	Not done	Not done	
GB 8B13	Tissue culture 7th pass		Not done	Not done	3 months

at 2 months of age and have now been on experiment for 15 months.

CONCLUSION

The reproduction of the scrapie syndrome by animal inoculation is at present the sole criterion of the presence of the agent in tissues or in culture. This has not been achieved and there is at present no evidence from our work for the isolation of an agent which can be directly associated with the exciting cause of scrapie in its classical form, in mice, sheep or goats. However, the ability of mouse-scrapie to survive at least one subculture perhaps constitutes grounds for optimism and two very important characteristics of the agent must be borne in mind. Sheep scrapie agent on first transmission to a new host, may have an extended incubation period. For example, Cheviot agent via Welsh Mountain sheep required 15 months to produce the disease in goats, (Pattison (4), 1957; Gordon and Pattison (5), 1957) and transmission of scrapie to rats was protracted and symptomless on first passage (Chandler and Fisher (6), 1963). Secondly, scrapie agent from some sources cannot so far be transmitted to another species; Cheviot or half-bred agent has not been adapted to mice and Suffolk agent is difficult to transmit to Cheviot sheep. These interspecies and strain peculiarities are discussed by Zlotnik (7) (1964) at this conference.

It is surprising that so few agents capable of transmission to experimental animals or of causing recognisable cytopathogenicity in tissue cultures have been recovered from sheep in the many stages of experimental scrapie which have been investigated. We have experienced no difficulty in isolating orthodox agents, such as louping-ill, contagious pustular dermatitis and virus abortion of ewes and in working with them in tissue culture. Similarly a number of agents have been recovered from mice which have proved to be associated with the colony and not with scrapie. During our work with tissues from young lambs and with old sheep, with and without the connotation of scrapie,

we have observed certain peculiarities. Some viruses yield much lower titres on sheep tissue cultures than on those from other species—e.g., vaccinia. In this connection, too, louping-ill virus has little or no overt pathogenicity for sheep kidney tissue cultures but the virus yield can be shown to be as high as that from pig kidney.

It will, therefore, be no surprise to some people here that we may be faced with a complex situation in respect of the role of agents which are now being isolated from sheep. The questions of latency of sheep viruses is very pertinent and it is likely that the environment in which much of our work has been conducted may prove to be quite unsuitable for the future. We are concerned here today, however, with the possible role of a particular agent or agents vis-a-vis scrapie. We are confident that in the workshop discussions we will learn much of value to our problems.

REMARKS BY J. G. BROTHERSTON FOLLOWING COLOR TRANSPARENCIES TO ILLUSTRATE THE NATURE OF THE CYTOPATHOGENIC EFFECT

The failure to demonstrate in tissue culture an agent directly associated with scrapie is still not incompatible with a virus etiology because many combinations and permutations of tissue culture techniques yet remain to be attempted. Dr. Koprowski has just described elegant techniques using rabies virus which are most encouraging to similar experiments with scrapie.

After a long series of negative results in tissue culture, one tends to associate the infectious nature of scrapie with reports of unorthodox agents or situations. I mention, for example, the Kappa factor of Paramoecium, and the possible existence of infectious nucleic acid or lipid-coated virus, resistant to heat. Even the suggestion of an agent arising de novo as a result of the passage of brain material from animal to animal cannot be overlooked. Such speculations tend to be unprofitable and sterilizing after a time because they provide very difficult model hypotheses with

which to experiment. It is, therefore, most stimulating to my colleagues and myself to work with an agent which appears to behave in an orthodox fashion. Its relationship to scrapie at this stage can only be speculative, but so few virus agents have been recovered from sheep that the characterisation of this one is of considerable interest.

Where then do we stand with the cytopathogenic agents that I have just described? We are examining the following possibilities:

1. They are latent contaminants of the pig kidney tissue culture system.

2. They are picked up as a result of contamination from the people involved in the work. So far both of these have been ruled out, as control cultures have failed to show similar effects.

3. They are the result of using material from natural cases rather than experimental cases.

4. We also consider that they may be the consequence of scrapie brain damage and of secondary significance to the exciting cause. Damage to the blood-brain barrier in scrapie has been postulated by Mr. Pattison.

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ADDITIONAL NOTE OF EXPERIMENTS IN 1965

Since this paper was read at the symposium, further work has shown that cytopathic effects similar to those described have arisen in pig kidney monolayers which have never been exposed to tissues from sheep, goats or mice, whether scrapie-affected or not. Under these circumstances, it is now the opinion of the authors that the effects described in relation to scrapie must be regarded as most likely due to latent contaminants in the pig-kidney tissue culture systems.

DISCUSSION

CASALS: In regard to your failure to reproduce the disease in sheep by inoculating the agent maintained in tissue culture there is a hemorrhagic disease of deer of which there are two strains in this country. The New Jersey strain has been adapted both to tissue culture, to HeLa cells, and to mice after years of trying. Now, as I understand—the work was done by Mettler and Shope—after a number of passages, four or five, in mice, the strain was put back into deer and they failed to reproduce the disease; however, the deer developed antibodies against the mouse strain. The point is this, whether you could take the sheep that had been inoculated with your tissue culture and see whether that serum would protect against your natural scrapie or not.

POSKANZER: Could you clarify the point about whether scrapie is transmitted from sheep to sheep naturally, or not?

BROTHERSTON: There is good evidence for natural transmission but it is from sheep to goats. We have no good factual evidence of scrapie spreading from sheep to sheep. A number of young goats were put in with successive numbers of Suffolk sheep suffering from scrapie and four out of five went down. But it took 2 to 3 years for the first case to go down and they were in constant contact with successive cases.

POSKANZER: It is transmitted from goat to goat?

PATTISON: There is a tremendous amount of discussion and argument on this whole question. Heredity, without doubt, in sheep, is enormously important, but there is a certain amount of circumstantial field evidence that contact spread in sheep may occur from time to time in certain very rare circumstances. This is about the situation. As Mr. Brotherston said, at Moredun Institute a group of goats put in contact when they were very young with natural cases of scrapie in sheep did in fact develop scrapie; but putting, as we have done at Compton, scrapie-affected goats with normal goats, as well as sheep, for many years, we have never managed to get it to go.

BROTHERSTON: We have a second experiment operating between Compton and Moredun to repeat this. At Compton they are putting them in contact with experimental scrapie and at Moredun with natural scrapie, and we have a third group elsewhere with very little chance of contact, so we should get the answer somewhere, if we live long enough.

Observations on the Agent of Scrapie

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The causal agent of scrapie has not yet been characterized nor indeed has it been placed in a definite category. Because of the numerous difficulties encountered in work on this agent progress has been slow. Most serious of these difficulties are perhaps that detection of activity is still dependent on animal inoculation in which the incubation period is long, and the marked affinity which the agent seems to have for tissue debris.

It is known that the disease can be transmitted experimentally not only from sheep to sheep, but also to the goat, mouse, hamster and rat. Filtrates from membranes of A.P.D. of 100 m μ have been found active. These findings have led to the conclusion by most workers that scrapie is caused by a virus. On the other hand its many unusual properties have led Pattison (1) to state that if the agent is a living virus it is likely to be a virus of a kind as yet unrecognized. However, as more information is being gathered about other recognized viruses, these differences are becoming less marked.

Work which we have carried out at Compton has been concerned mainly with attempts to cultivate the agent of scrapie in tissue cultures, whole animal experiments and a search for a serological reaction, and some of the results obtained are reported briefly in this paper.

Tissue Culture Experiments

In our tissue culture studies the greater part of our activities has been concerned with cultivation of mouse adapted scrapie (Chandler strain) in mouse embryo cells. More than 20 series of passages have been made in this way. As inoculum we have used 1/100 dilution of mouse brain suspended in medium and added either to established monolayers or to suspended cells.

Passage has usually been made at 14 day intervals. During this work we have looked for cytopathic effects, failing which the cultures have been inoculated into mice; this has usually been done at about the fifth passage level.

Typical scrapie (i.e., both clinically and histologically) has been produced only in mice which received material from the first passage level.

In one series only were obvious cytopathic effects observed in our cultures. The changes in the cells were seen from the fourth passage level. Stained preparations of coverslips showed bodies resembling mycoplasma and we have succeeded in cultivating a mycoplasma from the cultures. Material from this series was inoculated into mice at the 5th, 6th, 7th, 11th, and 16th passage levels. In most of the mice which were under observation up to 12 months no reaction was observed but in the case of the seventh passage the mice were held 14 to 18 months before they were killed. In some of these mice lesions resembling scrapie were seen in histological sections (fig. 1), differing only, we believe, in degree from characteristic lesions described by Pattison and Smith (2). Subinoculation from brains of these mice into other mice have not produced typical scrapie in a 12 months period but we have some still under observation.

Similar changes have been seen in histological sections of mice injected as infants with tenth serial passage material from another series and killed from 10 to 12 months later. Some of these mice are still under observation but at present the results obtained are: four apparently negative, two mild changes and one extensive degenerative change indistinguishable from scrapie.

These results are most interesting and while we have been encouraged to believe they may be caused by a

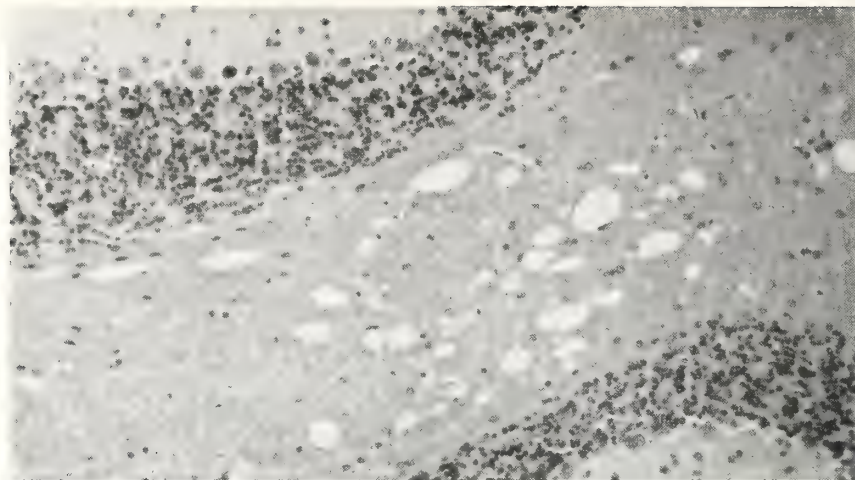


FIGURE 1.—Extracellular vacuolation in white matter of mouse cerebellum. Mouse was inoculated 18 months previously with 10^{-7} dilution of seventh passage material.

TABLE I.—Lesions in Mice Inoculated IC With Material Passaged in Mouse Embryo Cells

Passage level	Dilution	Months after inoculation	Lesions			
			N	+	++	+++
I. 5	Undiluted	8-12	11			2
6	Undiluted	10-12	5			
7	10^{-3}	14-18	3	4	2	
	10^{-4}	14-18	1	3	6	
	10^{-5}	14-18	3		1	
	10^{-6}	14-18		2		
	10^{-7}	14-18		1		
11	Undiluted	10-12	5			
16	Undiluted	10-12	5			
II. 10	Undiluted	10-12	3	4	2	1

N=no lesions seen.

+ = slight lesions of encephalopathy.

++ = moderate lesions of encephalopathy.

+++ = lesions indistinguishable from scrapie.

modified form of scrapie, there are other possible explanations such as old age, carry-over of original inoculum, or even artifacts.

Of the many goats (± 80) which we have inoculated with tissue culture preparations we have observed reaction in only 4. One injected with material from the 10th serial passage of kid kidney cells, i.e., cell line culture, reacted and showed typical lesions. However, two goats which received fifth passage material did not react and our conclusion was that this reaction resulted from agent carried over with the transfers.

In two goats inoculated with material from the fourth passage level in kid kidney cultures and one

which received sixth passage ME cultures, reactions have been observed in which the lesions have been characterised by an exaggerated "status spongiosus." We do not know what relationship this lesion bears to scrapie, but in the two instances in which sub-inoculations have been made to other goats, no reactions have as yet occurred after observation periods of 18 and 20 months.

Other work has been concerned with the observation of brain explants in plasma clots. Here we have been able to repeat the observation of Field and Windsor (3) when mouse brains were used but not in the case of goat brains.

Whole Animal Experiments

Mainly with the object of gaining some idea concerning the optimum time for harvesting mouse brains for our tissue work, growth curves were made of the agent in mouse brain. Two separate trials were made. In each a group of mice was inoculated with supernatant fluid from 10 percent scrapie mouse brain suspension. Mice were killed at intervals and the activity of the agent in their brains was determined by titration in mice. Lesions were demonstrable histologically after 56 days, while symptoms were first noticed after 112 days; these times coincide closely with those reported by Pattison and Smith (1963).

The results of these experiments are shown in the graphs (fig. 2). From these it appears that there is a rapid removal of activity from the brains of inoculated mice probably to other parts of the body. The residual activity remains fairly constant for a period of about 11 days after which there appears to be a steady

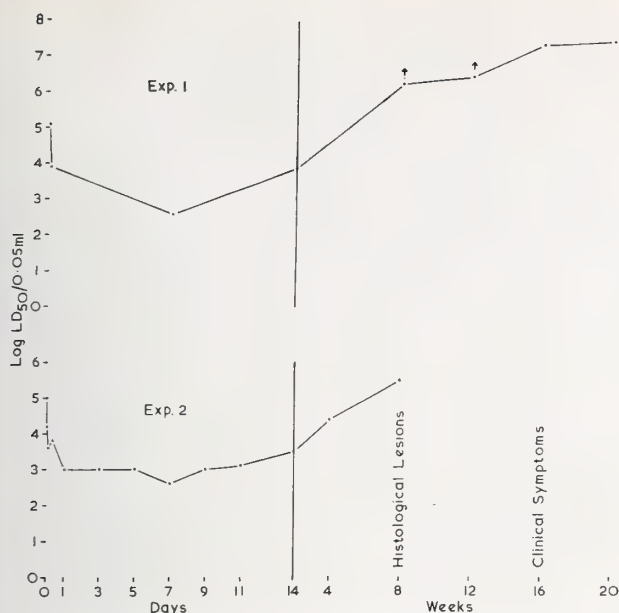


FIGURE 2.—Infectivity of mouse brain at intervals after intracerebral inoculation with scrapie agent.

rise in activity apparently at the rate of 10-fold rise each 3 weeks. These results have encouraged us to believe that multiplication of the agent can occur in the brain itself.

Size of the Scrapie Agent

Many years ago Wilson, Anderson and Smith (4), working at Moredun Institute, showed that this agent could be filtered through a membrane of APD 410 m μ . Later it was reported by Stamp (5) that a filtrate obtained from a membrane of APD 210 m μ proved positive.

Working with mice, Eklund, Hadlow and Kennedy (6) found that brain suspension, clarified by Seitz filtration, was infective after filtration through a membrane of APD 100 m μ , but not when a membrane of APD 30–100 m μ was used; they believed that the particle size was in the order of 50 m μ .

Apparently Wilson realized that the agent might well be considerably smaller than this filtration experiment had indicated, and he carried out work on the effect of high-speed centrifugation. Wilson found that the supernatant fluid obtained after 40,000 r.p.m. for 2 hours was active. Although this result could be accounted for by technical considerations it could also mean that the agent failed to sediment completely at this force. It was this consideration that stimulated Pattison and Sansom (7) to attempt dialysis of infective preparations. Their finding of activity outside the dialysing bags does not give precise evi-

dence concerning the size of the agent, although the inference is that it may be very small. Apart from technical considerations which they discuss, it is apparent that only a small proportion of the agent was found outside the bag. Of necessity most work on animal viruses is concerned with the behaviour of the bulk of an agent and little is known of what occurs on the fringes. Nevertheless, in these various experiments there is an indication that the particle size of the scrapie agent may be extremely small.

These results, and those of Hunter and Millson (8) and Mould, Smith and Dawson (9) on the activity of cell fractions, have led to the belief that the scrapie agent is relatively small and that the difficulties that have been encountered in determining its size may be due to its firm adhesion to debris. Before more definite information can be obtained on such properties as size, a method must be devised for separating the agent from cellular components. Of our numerous attempts to do this the most promising has been treatment with Arcton-113. Our early results with this substance proved variable, but it now seems that provided the manipulations are carried out at low temperature (2° to 4°) reasonably satisfactory preparations can be obtained (table IIc, Millson and Hunter, personal communication).

The results of these experiments are shown in table II.

TABLE II.—Effect of Arcton-113 on Activity of Scrapie in Mouse Brain Suspension

Treatment	Titre	N ₂ mg/gm.	Percent clearance
a. Control	6.5+	23.0	60
1 x Arcton	6.5+	9.2	
b. Control	8.1	21.6	56
1 x Arcton	6.8	9.6	
2 x Arcton	5.1	5.8	
3 x Arcton	4.4	4.0	
c. Control	5.7	19.9	64
1 x Arcton { Super	7.2		
Pellet	6.2	1.1	
2 x Arcton Pellet	5.5	0.22	

Using Arcton-treated scrapie mouse brain we have commenced a series of filtration experiments with Gradocol membranes prepared at the Wright Fleming Institute. In the first experiment which is now completed, the activity of the stock material proved disappointingly low (10^{2.5}LD₅₀/0.05 ml). However, cases of scrapie have appeared in 1 of 20 mice inoculated with material from a membrane of APD 54 m μ ,

but none from a membrane of APD 21 $m\mu$. We are awaiting the results of other experiments in this series since it appears the scrapie agent may be extremely small being probably less than 40 $m\mu$.

Enzymic Treatment

To examine the effect of various proteolytic enzymes on the scrapie agent 20 percent suspensions in buffer of infected mouse and goat brains were prepared. After light centrifugation these were mixed in equal parts with buffered enzymes or with buffer only. The mixtures were held at 37° for 3 hours then at 65° for 30 minutes. (Method of Caspary and Field (10).) They were then centrifuged lightly and titrated in mice or inoculated into goats by the intracerebral route. The results of these experiments are summarised in table III.

TABLE III.—Effect of Enzymes on Activity of the Scrapie Agent

Host	Treatment	LD ₅₀ /0.05 ml.		
		Pepsin	Trypsin	Ficin
Mouse	Enzyme	2.5	6.2	6.2
	Buffer only	2.5	6.9	
Appearance of scrapie in months				
Goat	Enzyme	NR; NR	10; 11	10; 10
	Buffer only	NR; NR	10	

NR=No apparent reaction.

Crystallised Pepsin Armour 0.5 mg./ml. in 0.1M acetate buffer pH 4.2.

Crystallised Trypsin Armour 0.5 mg./ml. in 0.1M phosphate buffer pH 8.

Crude Ficin L. Light & Co. 0.1 mg./ml. plus B.A.L. (Di-mercaptopropanol) 0.1 mg./ml. in phosphate buffer pH 8.

From these results it appeared that under the conditions of the experiment the various enzymes tested had no apparent effect on the activity of the agent. Decrease in activity in the acetate buffer was presumably due to precipitation of the agent.

Stability at Various pH Levels

The stability of the agent at various pH levels was examined. Equal volumes of supernatant fluid obtained by light centrifugation of a 2 percent suspension of infected mouse brain were mixed with a variety of buffers. The suspensions were held overnight at 4°, the pH was tested and the titres measured by mouse inoculation. The results of this experiment are shown in table IV.

TABLE IV.—Effect of Various pH Levels on the Activity of the Scrapie Agent

pH	Buffer	Titre
2.1	N/100 HCl	6.2
3.9	Citrate buffer	6.4+
5.8	Citrate buffer	6.5+
7.7	Tris buffer	6.5+
9.8	Tris buffer	6.0
7.0	Saline control	6.5+

While end points were not obtained in all titrations, it did appear the effect of pH was slight other than that at pH 2 and 10. Examination of the protocols showed that at these two levels the onset of symptoms was somewhat delayed indicating the differences shown are possibly significant.

Serum Neutralisation Tests

A series of neutralisation tests were carried out in which a variety of sera from normal and infected animals were mixed with 10-fold dilutions of infected mouse scrapie brain suspension. After 2 hours at 37° and overnight at 4° the mixtures were injected into mice. The results of these experiments are shown in table V.

TABLE V.—Serum Neutralisation Tests on Scrapie Agent

Serum	LD ₅₀ /0.05 ml.
Normal mouse	6.5+
Scrapie mouse	6.5+
Normal goat	6.5+
Scrapie goat	6.0+
Hyper I goat (scrapie brain)	6.5+
Hyper I goat (normal brain)	6.5+
Recovered Swaledale	6.5+

Unfortunately end points were not obtained but analysis of the protocols did not reveal any apparent differences.

SUMMARY

It is not possible to draw definite conclusions from the results which have been presented from work which is still in progress. However, there are indications that the agent has a particle size of less than 40 $m\mu$, it is resistant to a wide range of pH levels, and is not affected by the proteolytic enzymes which were examined. Attempts at clarification of suspensions

of infected mouse brains have not been satisfactory and more work on this aspect is required. It is not known whether the agent can be propagated in tissue culture but it appears that if it does multiply, this is accompanied by a decrease in pathogenicity making detection of activity most uncertain.

No evidence of neutralising antibodies could be detected under the conditions of the test in serum taken from advanced cases of scrapie in mice or goats.

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Evidence of the Presence of Scrapie in Cell Cultures of Brain¹

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Of the many handicaps attending experimental efforts to produce useful information about scrapie, the remarkably long incubation period and the necessity of using the natural host, sheep, seems to have constituted the greatest block to rapid progress. The physical requirements and consequent expense of conducting research with large numbers of sheep are great. The difficulty is accentuated by the period of time involved in an experiment in which a multitude of other variables make the results more difficult to interpret.

The incubation period is rarely less than 5 months, and has been recognized as having no definable maximum period. Compounding these difficulties is the variation in susceptibility among breeds of sheep. Similar comments are applicable to the use of goats with the possible exception that goats are more uniformly susceptible.

The transmission of scrapie to mice (1, 2), rats (3), and hamsters (4) has reduced the difficulties of working with scrapie in substantial measure. The maximum incubation period has been significantly reduced and the minimum has been slightly reduced.

Many efforts have been devoted to the demonstration of antibodies against the agent of scrapie and all have failed (5, 6, 7). Differential centrifugation studies (8, 9) were reported to reveal that the agent is particulate and self-replicating. Nevertheless, without an immunological basis for diagnosis the disease remains a clinico-pathological entity. Diagnosis of the disease in animals is made on the basis of observations of signs of the disease and the interpretation of histopathological preparations of the brain and spinal cord. In mice, one of the most important pathological

changes is a marked hypertrophy and possibly hyperplasia of astrocytes in the brain and spinal cord. In addition there is a generalized spongy appearance of the brain most often observed in the medulla and in the superior and inferior colliculi.

We reported (10) transmission of scrapie to grade lambs by subcutaneous inoculation of material from a pure-bred Suffolk sheep of an Indiana flock affected with scrapie. From one of the experimentally produced cases of scrapie, monolayer cell cultures were prepared and had been subcultured six months at the time of the report.

The morphological differences of the cells and differences in colonial characteristics when compared with similar cultures from an unaffected sheep aroused interest in the possible contribution of cell culture techniques to research on the agent of scrapie.

This report presents amplification of the previous report. It is concerned with efforts to demonstrate the presence of scrapie virus in cell cultures from the brains of an affected sheep, affected mice, and in cell cultures from an unaffected sheep which had been exposed to fluids from the cell cultures from the affected sheep. This we sought to accomplish through the inoculation of mice with harvested cell culture fluids.

MATERIALS AND METHODS

Scrapie Virus

1. Portions of the brain of a "scrapied" purebred Suffolk sheep from a flock in Indiana served as the source of scrapie virus. The virus was demonstrated by experimental induction of the disease in grade sheep by subcutaneous exposure to the supernatant fluid of a centrifuged 10 percent suspension of the sheep brain.

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2. A strain of scrapie virus from the Agricultural Research Council Field Station, Compton, Berkshire, England, was used to infect mice from which whole brain cell cultures were made. It was used at the fifth serial passage in mice.

Mice

White Swiss and C57 Be/6 JAX mice obtained from the Roscoe B. Jackson Memorial Laboratory at Bar Harbor, Maine, were used to develop random bred colonies of each breed.

Preparation of Cell Cultures

1. Sheep Brain

The method used to prepare monolayer cell cultures from the thalamus and midbrain of sheep was reported previously (10). The cultures were originally set in Leighton tubes and T9 flasks. Subsequent serial subcultures were made from individual tubes. Some tube cultures were subcultured more often than others. The range at 30 months when they were lost was 23 to 34 subcultures. The cells could be maintained in ordinary soft glass prescription flasks, soft glass tubes, or borosilicate glass tubes. Transfer of cells in subcultures was accomplished with the use of 0.25 percent trypsin in the common fashion.

The initial medium used was 79 parts of Eagle's Basal Medium (EBM) containing 350 mg./percent glucose, 20 parts of calf serum, and 1 part SPTN (streptomycin 100 mgm., penicillin 100 units, tetracycline .01 mgm., and nystatin 50 units) per ml. of Hanks' balanced salt solution (BSS). Subsequently, the use of nystatin was discontinued and the serum component was reduced to 10 parts and from time to time some of the cultures were changed to fetal calf serum, lamb, rabbit, or human serum.

2. Mouse Brain Cell Cultures

Whole brains from normal mice or from mice affected with scrapie were prepared in much the same fashion as were the sheep cell cultures. Brains were cut into small pieces rinsed with balanced salt solution (BSS) and added to a Rappaport trypsinization flask containing about 30 ml. of 0.20 percent trypsin at 37° C. The mixture was stirred for 2½ to 3 minutes with a magnetic stirring device. The fluid was poured off through cheese cloth into a large flask containing about 150 ml. of Rinaldini's solution at room temperature. The chunks of brain were retained in the Rappaport flask. Additional 0.20 percent trypsin at 37° C. was added and the process was repeated 3 or 4 times.

The trypsinized cells and fragments were centrifuged lightly, resuspended in BSS, centrifuged and the process repeated once more. The brain material was then resuspended in complete medium. Dilution of the cells was usually achieved by estimation because the amount of debris present precluded the possibility of achieving a satisfactory cell count in a hemacytometer. On one occasion a cell count was possible and cultures were initiated with about 600,000 cells per ml. The cell suspension was then distributed to the vessels for incubation.

The medium was allowed to remain on the cell cultures for 3 days without disturbing the cultures. At that time about half of the medium was removed and replaced with fresh medium.

3. Exposure of Cell Cultures from the "Normal" Sheep to Fluids from Cell Cultures from the "Scrapied" Sheep

Some cell cultures from the brain of the sheep not affected with scrapie were exposed to fluids harvested from the cell cultures from the sheep affected with scrapie. Fluid transfers were initiated when the cultures were about 4 months of age. At five successive changes of medium, which occurred twice each week, harvested fluids were transferred from the "scrapie" cell cultures to the "normal" cell cultures. The amount transferred each time was 0.1 ml.

Maintenance of Cell Cultures and Storage of Harvested Medium

The medium on the cell cultures was changed twice per week with exceptions based on observation of the cell cultures.

The medium removed from the cell cultures was saved occasionally, sealed in ampoules, and stored at about -35° C. Selected harvests were later used as inoculums for mice.

Mouse Inoculations

Both suckling and weaned mice were inoculated intracerebrally with cell culture fluids. The weaned mice received about 0.03 ml. and the suckling mice about 0.01 ml.

Thirteen groups of mice were inoculated. Eight of the experiments are incomplete at this time although useful information seems to be available from all. All were inoculated with cell culture fluids harvested at various times from the cultures indicated in the following general descriptions of inoculums. These are described in table I of RESULTS.

A. Fluids harvested from cell cultures of the thalamus and midbrain of a sheep affected with scrapie.

B. Fluids harvested from cell cultures of thalamus and midbrain of a "normal" sheep but exposed to medium harvested from the cell culture from a sheep affected with scrapie after 4 to 5 months of culture.

C. Fluids harvested from primary cell cultures of whole brains of mice affected with scrapie. The Compton strain of scrapie virus was used.

D. Samples of medium from primary cell cultures of brains of mice not affected with the disease nor exposed to the virus.

Diagnosis of Scrapie in Mice

The decision of whether or not a mouse was affected with scrapie was based on signs of the disease, which have been well described, and histopathological changes in the brain and spinal cord (1, 2). Astrocytosis in the brain (1) and spinal cord was considered to be highly significant as was extracellular vacuolation of more or less generalized distribution in the telencephalon and mesencephalon (11).

RESULTS

A. Observations of Cell Cultures

1. Cells from the Brain of a Sheep Affected with Scrapie

During the first 2 weeks of culture the cells which survived adhered to the glass singly rather than in clumps or other aggregate forms and appeared to adjust themselves to the new environment more slowly than other types of cells, such as kidney cells for example. The dominant forms were bipolar or tripolar perikaryons densely enclosing the nuclei. About the middle of the second week nearly all of the remaining cells appeared to be astrocytes. The cytoplasm began to spread out from the juxtanuclear zone (fig. 1) in thin membranes and to extend from the pseudopodic extensions laterally to broaden them. Cytoplasmic thin membranes filled in between the extensions (fig. 2) and binucleated cells were observed (fig. 3). Mature astrocytes were found with many cytoplasmic extensions (fig. 4). In general the cytoplasm continued to increase in dimension during the third week. About the 18th day the cells had become numerous enough and possibly altered enough to associate loosely in colonial aggregates which later progressed to cell sheets (figs. 5, 6). The cells contained eosinophilic granules in the cytoplasm arranged about both the nucleus and a spherical juxtanuclear zone. The

margins of the cells were serrated and the membranous clear cytoplasm often showed evidences of pinocytosis. Some cells developed very long dendrites and fan shaped membranes emanating from the perikaryon (fig. 7). Many of the astrocytes continued to grow and amass a very large amount of cytoplasm which was often radially ribbed (fig. 8). Such cells were often binucleated with very large nuclei.

In the period from 22 to 40 days of culture, important characteristics of the astrocyte component became more apparent. Very large cells were seen (figs. 9, 10) in contrast to the common medium sized cells (fig. 11). Portions of cultures were formed by the large cells with membranous ribbed cytoplasm (fig. 12). During this period overlapping and "piling up" increased in intensity (figs. 13, 14, 15). Granularity of the cytoplasm was intense and with apparent fusion of the eosinophilic granules into inclusions. Multinucleation became quite common with cells having 2 to 5 and sometimes more nuclei (fig. 16). Amitotic division was suggested by observations of blebbed nuclei (fig. 17) and the presence of small nuclei satellite to large nuclei.

In the period from 3 to 8 months of culture a wide range of nuclear size was present. Bizarre nuclear forms were observed and multinucleation was of moderate order but rather high intensity (figs. 18, 19, 20).

2. Cells from the Brain of a Sheep Not Affected with Scrapie

Cultures of cells from the unaffected sheep were somewhat slow to multiply. At 40 days they appeared to reach the multiplicity achieved in about 18 days by similar cultures from the scrapie source. The cells were almost entirely astrocytes (fig. 21). In some, the cytoplasmic expansion was not highly developed (fig. 22).

Cellular size range during the next 2 months was relatively narrow (fig. 23). The nuclei were oval with the long axis parallel with the long axis of the cytoplasm. The margins of the cells were serrated. The cytoplasm was thin, clear, and homogeneous except for the juxtanuclear ring of eosinophilic granules. There was some limited overlapping and, as cultures became crowded, the cultures developed some piles of cells (figs. 24, 25, 26). Cytoplasmic ribbing radially from the nucleus was not highly developed. Multinucleation was present in somewhat lower order and significantly less intensity than in similar cultures from the scrapie source. After 8 months during which three serial subcultures were made, the characteristics of the cultures remained constant (figs. 27, 28).

3. Sheep Brain Cell Cultures Exposed to Fluids Harvested from Similar Cultures from a Sheep Affected with Scrapie

Cell cultures from the unaffected sheep exposed to fluids from cultures derived from the sheep affected with scrapie developed characteristics reminiscent of the features of the latter. The changes were first observed at 27 days. Multinucleation, marked overlapping of cells, bizarre nuclear shapes, increased range of nuclear size, decreased polarity of cells in the colony (fig. 29) constituted the changes considered to be involved. However, the changes were not observed to reach the order of difference from the unaffected sheep cell cultures that was observed in the cells from the scrapie source.

4. Cell Cultures of Whole Brains of Mice Affected with Scrapie

Comparatively, it has been our experience that cell cultures from mice affected with scrapie have been more readily established than from mice not affected with the disease. The progression of events in the establishment of the cell population in the culture are like those described previously for sheep. About the middle of the second week astrocytes are the dominant cell type and continue to be. The progression of membranous cytoplasmic development usually occurs in the third week. From this period on to 68 days, the longest period observed thus far, astrocytes from mice affected with scrapie have much in common with similar cultures from the sheep affected with

LEGENDS FOR FIGURES 1 TO 28.

FIGURE 1.—(a) and (b) Cells from "scrapied" sheep brain at 15 days. Astrocytes with developing cytoplasmic membrane formation expanding from perikaryons. (a) $\times 420$; (b) $\times 990$. Phase contrast.

FIGURE 2.—(a) and (b) Cells from "scrapied" sheep brain at 15 days. Astrocytes with membrane formation between cytoplasmic extensions. (a) $\times 260$; (b) $\times 420$. Phase contrast.

FIGURE 3.—Cells from "scrapied" sheep brain at 15 days. Binucleated astrocyte and a cell with prominent juxtanuclear eosinophilic granules, some of which appear to have fused into larger masses. $\times 650$. Phase contrast.

FIGURE 4.—Large fibrous astrocyte from "scrapied" sheep at 12 days in culture. $\times 460$. Phase contrast.

FIGURES 5 and 6.—Aggregations of astrocytes from "scrapied" sheep brain with nearly complete cytoplasmic development, about 3 weeks in culture. $\times 260$ each. Phase contrast.

FIGURE 7.—Astrocyte with long beaded dendrites and fan shaped cytoplasmic membrane extending from the perikaryon from the "scrapied" sheep brain at 18 days in culture. $\times 420$. Phase contrast.

FIGURE 8.—Protoplasmic astrocyte from "scrapied" sheep brain at 18 days with fibrous extensions having bulbous cytoplasmic terminations. $\times 420$. Phase contrast.

FIGURE 9.—Huge binucleated astrocyte from the "scrapied" sheep brain at 30 days in culture. (n) nucleus. $\times 260$. Phase contrast.

FIGURE 10.—Large astrocyte from the "scrapied" sheep brain at 30 days in culture. Ribbed cytoplasm is characteristic of such cells. $\times 260$. Phase contrast.

FIGURE 11.—Large astrocyte from the "scrapied" sheep brain at 25 days in culture with well defined cytoplasmic "ribs" may be compared with a medium sized astrocyte (mc). $\times 260$. Phase contrast.

FIGURE 12.—Large astrocytes in colonial aggregation with cytoplasmic overlapping. From the "scrapied" sheep brain at 39 days in culture. $\times 260$. Phase contrast.

FIGURES 13, 14, and 15.—Aggregations of astrocytes from the "scrapied" sheep brain at 30, 22, and 31 days respectively with overlapping, "piling up." Variable granularity and low order of colonial polarity. $\times 260$ (13, 14); $\times 650$ (15). Phase contrast.

FIGURE 16.—Multinucleation in "scrapied" sheep brain cells at 30 days in culture. $\times 490$. H. & E. stain.

FIGURE 17.—Blebbing of nucleus in huge astrocyte of "scrapied" sheep brain culture at 39 days. $\times 650$. Phase contrast.

FIGURE 18.—Wide range of nuclear size among cells of "scrapied" sheep brain cells at 240 days in culture. Multinucleation is of high frequency and relatively high order. $\times 310$. May-Grünwald Giemsa stain.

FIGURE 19.—High frequency of multinucleation in astrocytes of "scrapied" sheep brain at 150 days in culture. $\times 160$. H. & E. stain.

FIGURE 20.—Bizarre nuclei in large astrocyte of "scrapied" sheep brain at 94 days. $\times 650$. Phase contrast.

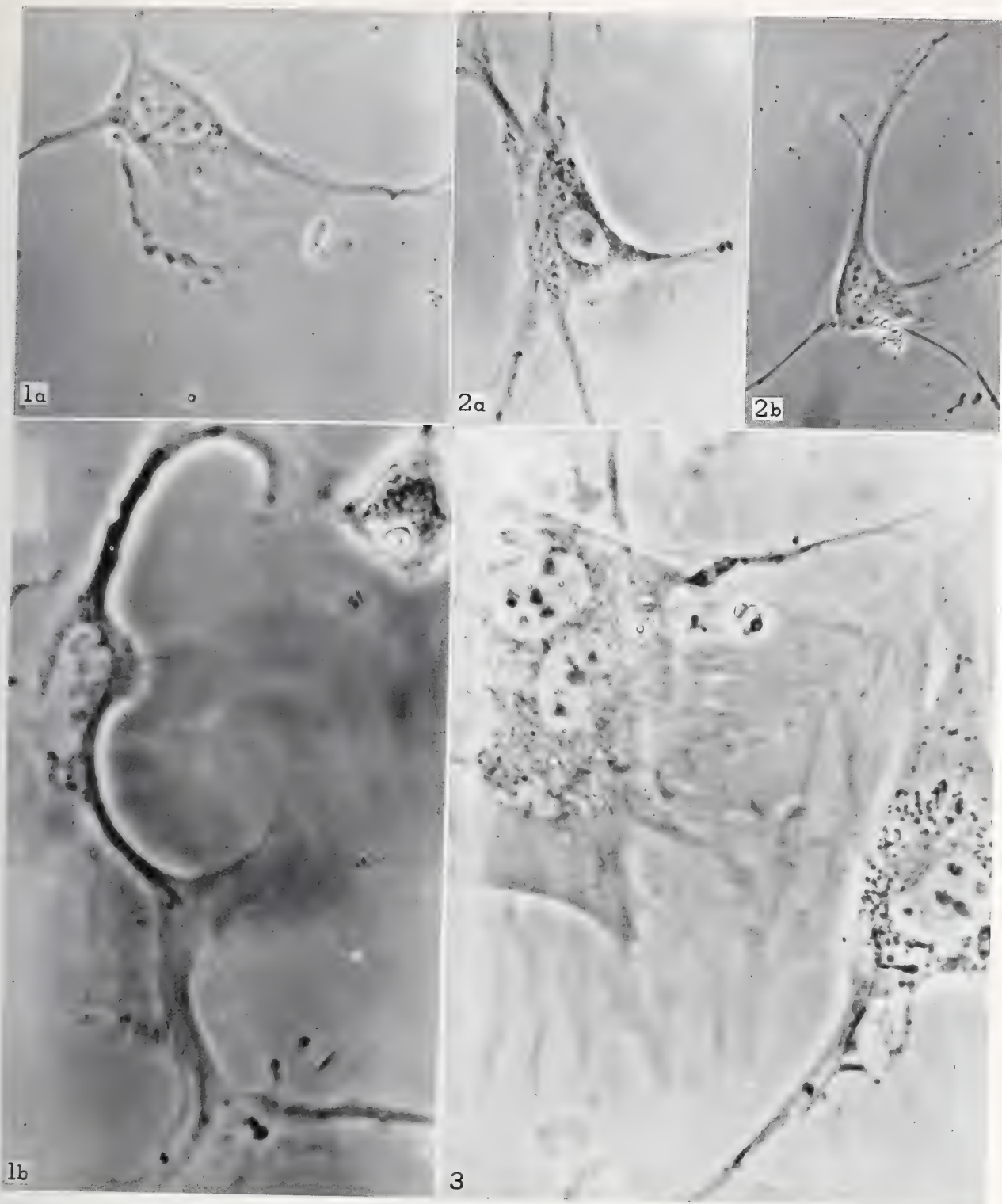
FIGURES 21, 22, and 23.—Astrocytes in culture from "normal" sheep brain at 40 (21, 22) and 49 (23) days. $\times 260$. Phase contrast.

FIGURE 24.—Astrocytes in culture from "normal" sheep brain at 94 days. Homogeneity of the cytoplasm and the regular arrangement of granules and juxtanuclear masses is to be noted. $\times 390$. Phase contrast.

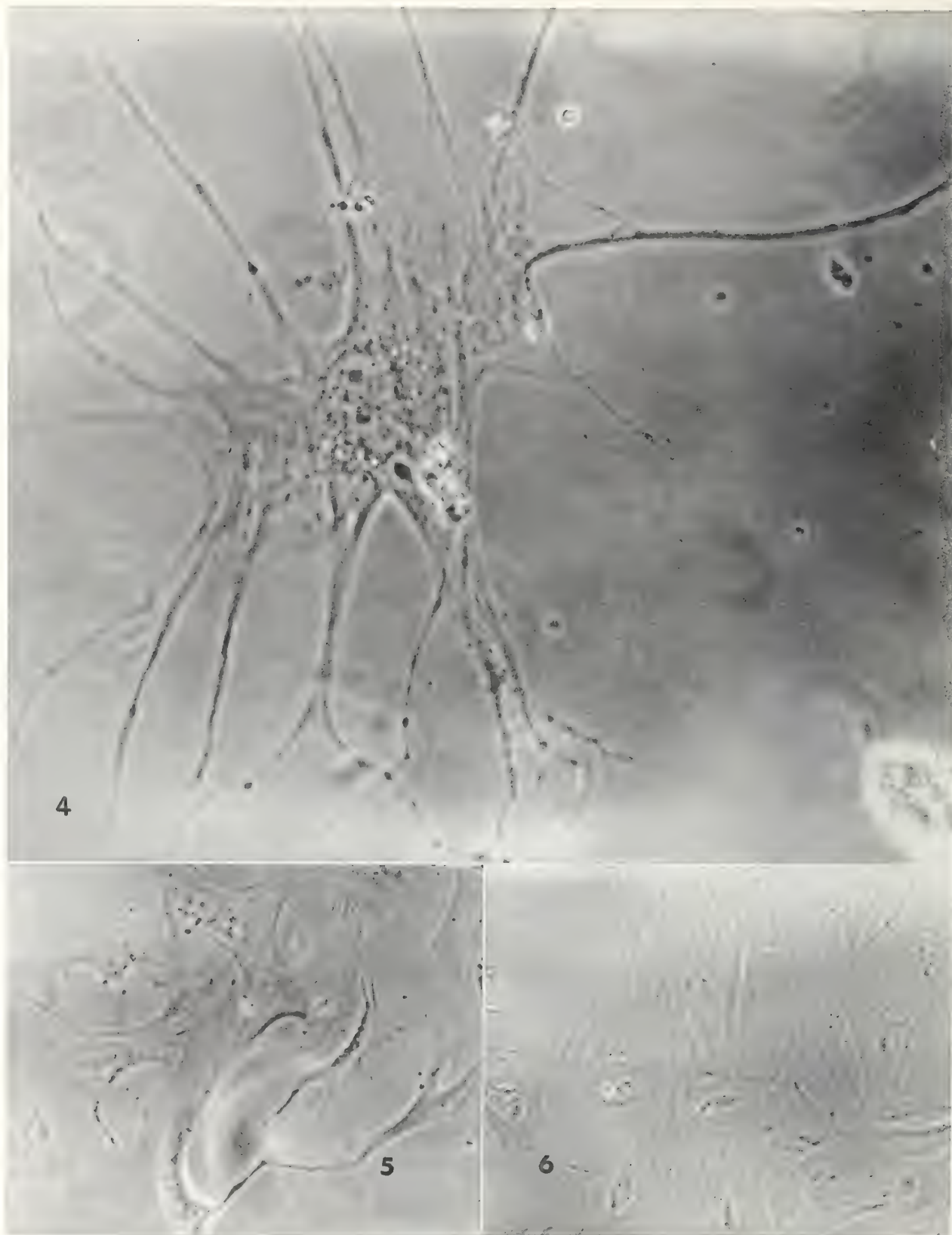
FIGURE 25.—Astrocytes in culture from the "normal" sheep brain at 54 days. Uniformity among the cells of this culture was striking in contrast to those from the "scrapied" sheep. $\times 990$. Phase contrast.

FIGURE 26.—Astrocytes in culture from the "normal" sheep brain at 49 days. The cells are rather uniform and there is some overlapping of cytoplasm. $\times 390$. Phase contrast.

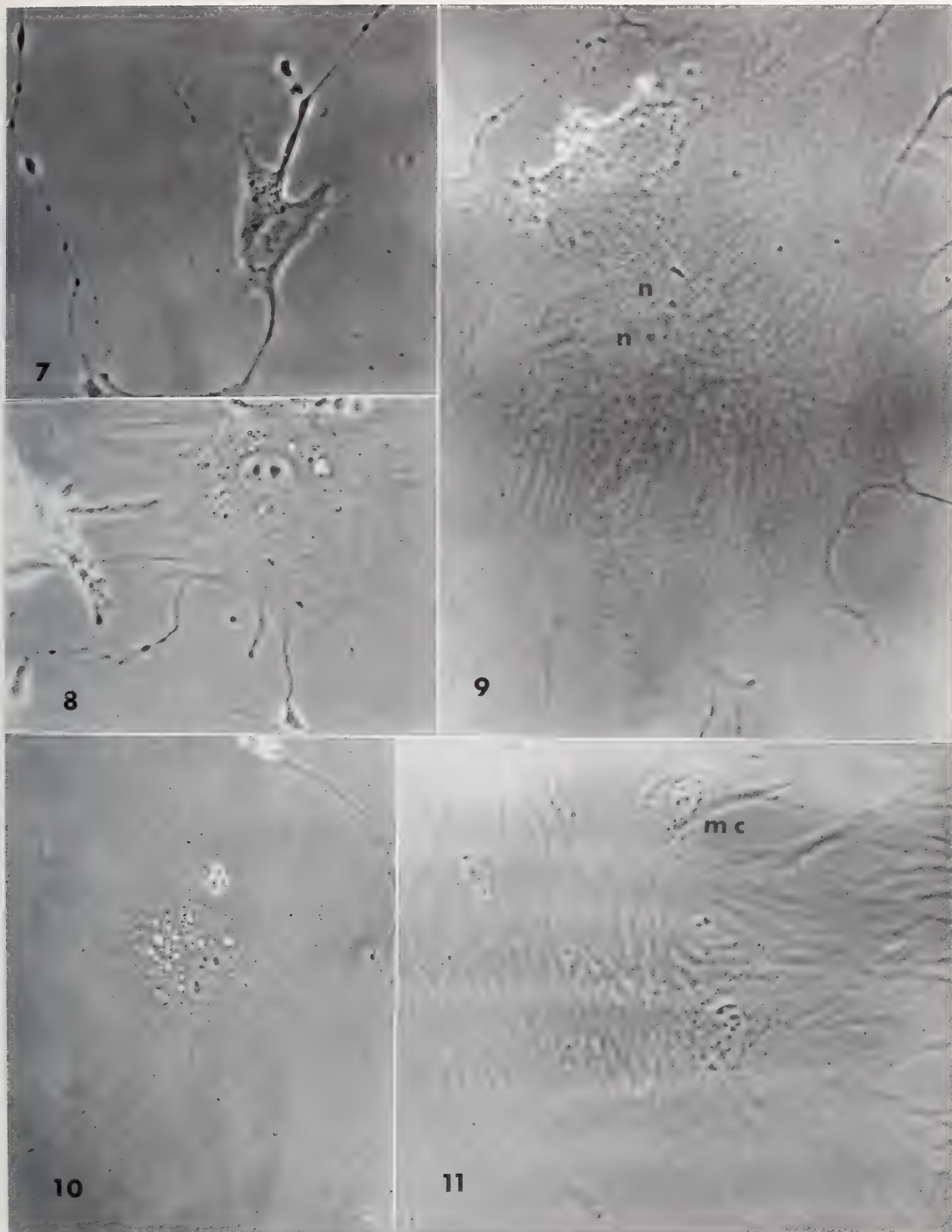
FIGURES 27 and 28.—Astrocytes in culture from the "normal" sheep brain at 224 days. $\times 30$ (27), $\times 390$ (28). May-Grünwald Giemsa stain.



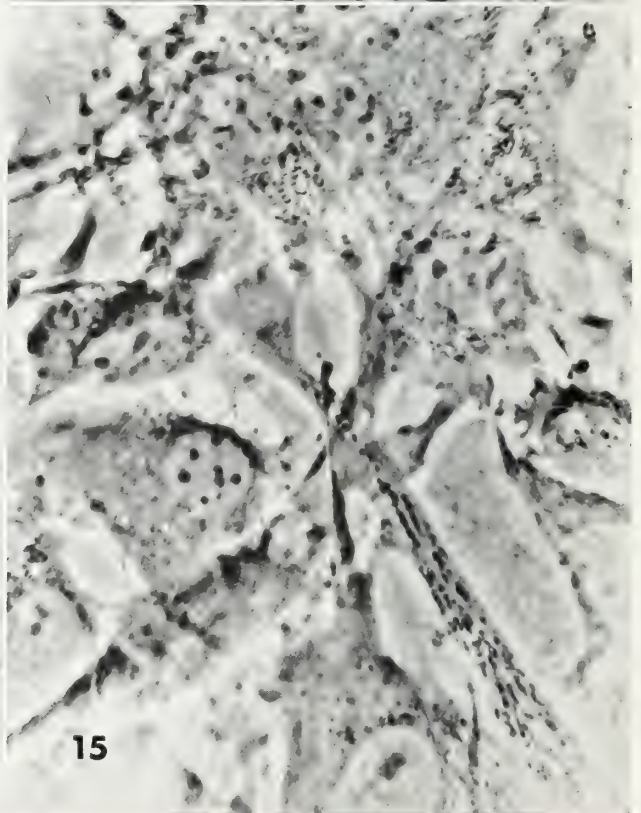
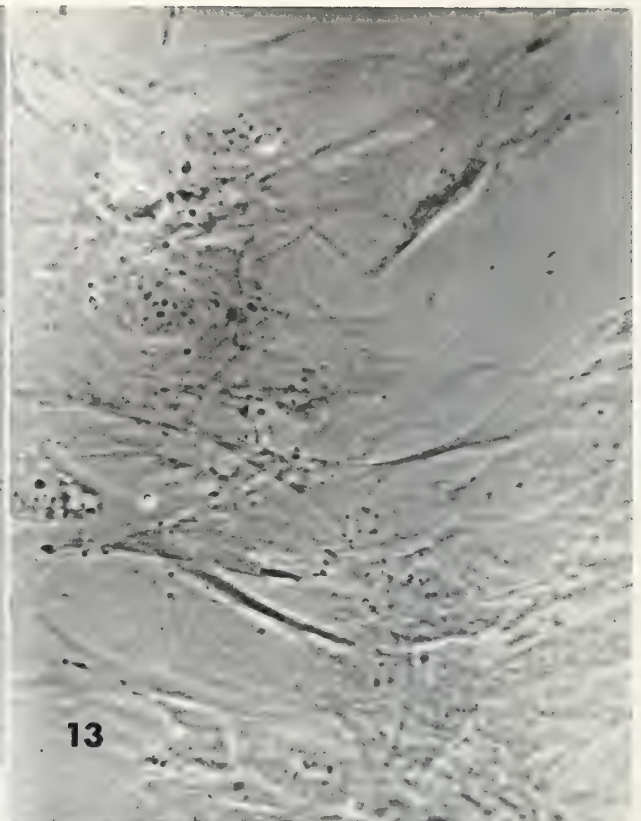
FIGURES 1a, 1b, 2a, 2b, and 3.



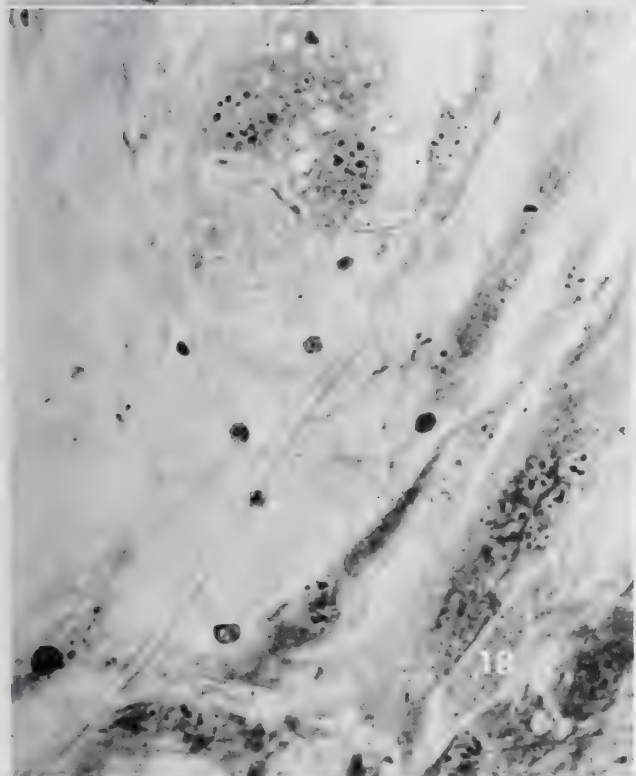
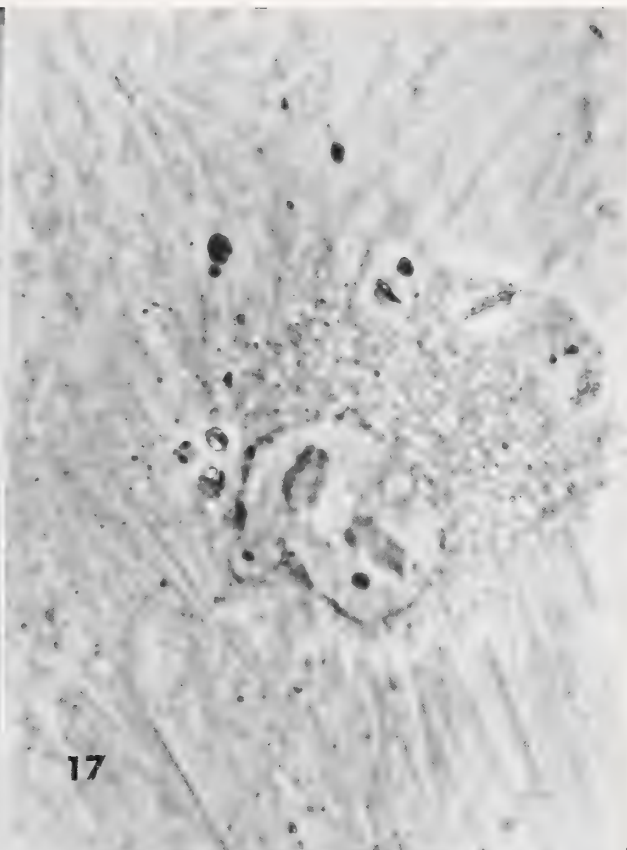
FIGURES 4, 5, and 6.



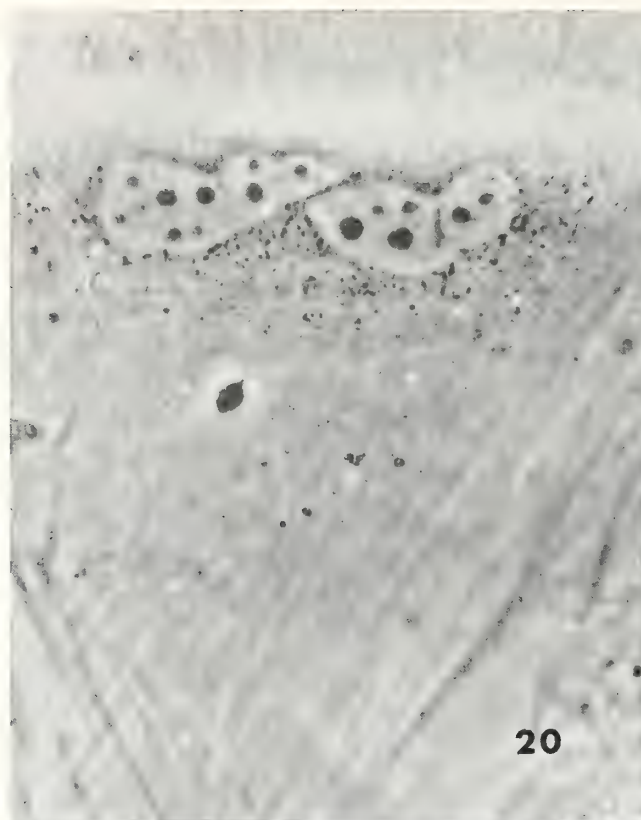
FIGURES 7, 8, 9, 10, and 11.



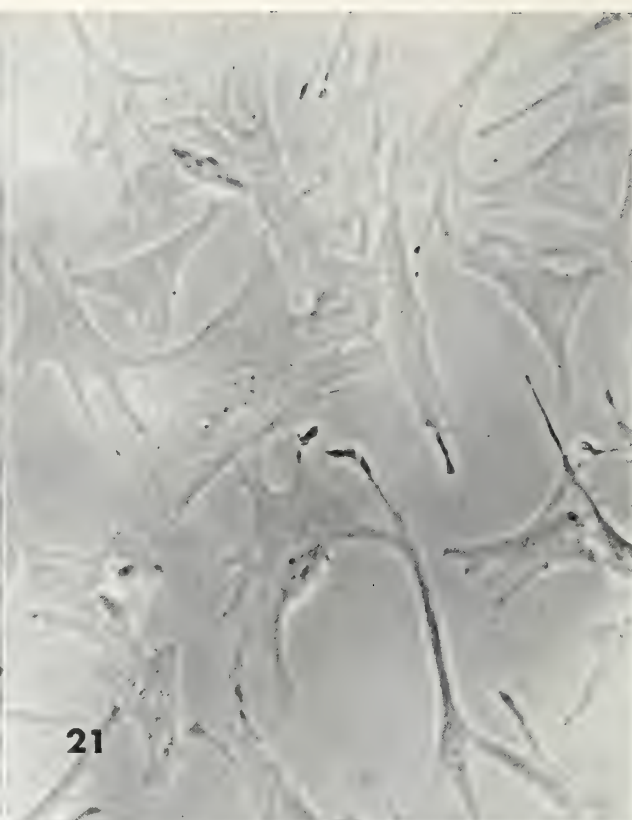
FIGURES 12, 13, 14, and 15.



FIGURES 16, 17, 18, and 19.



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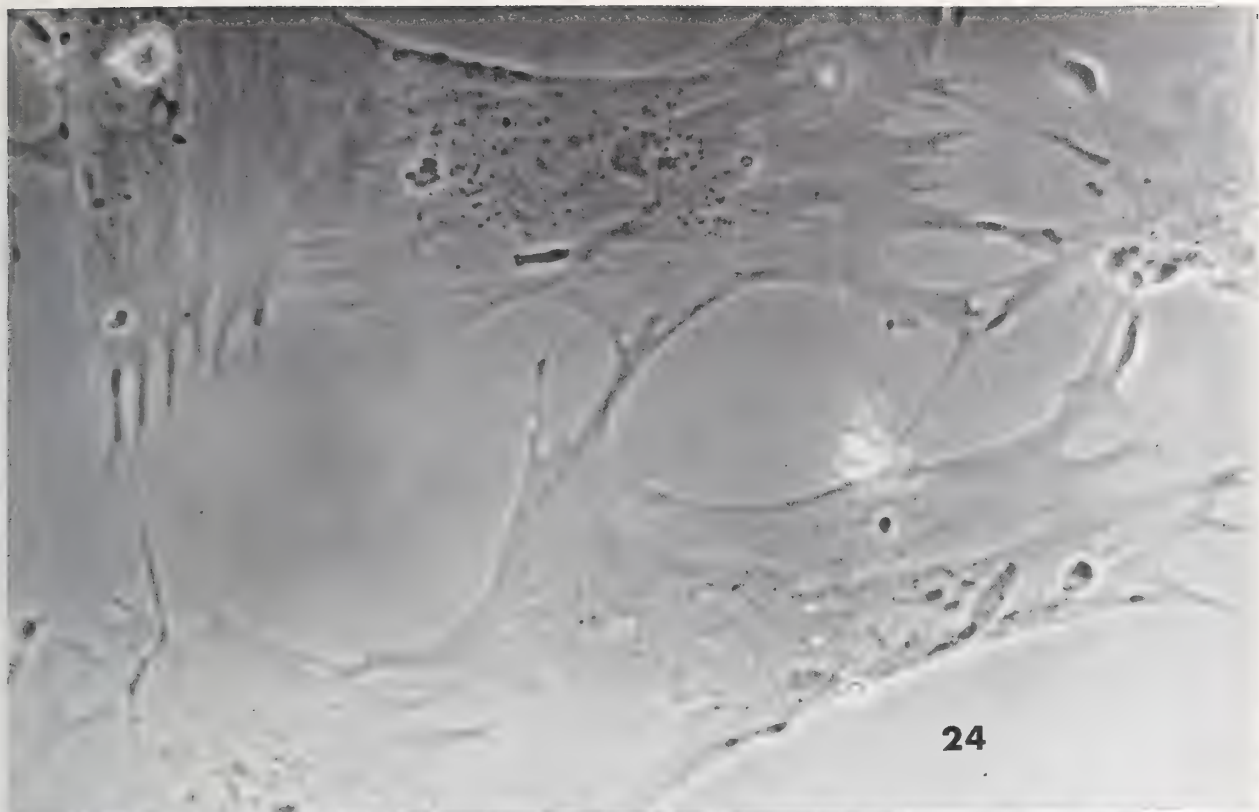


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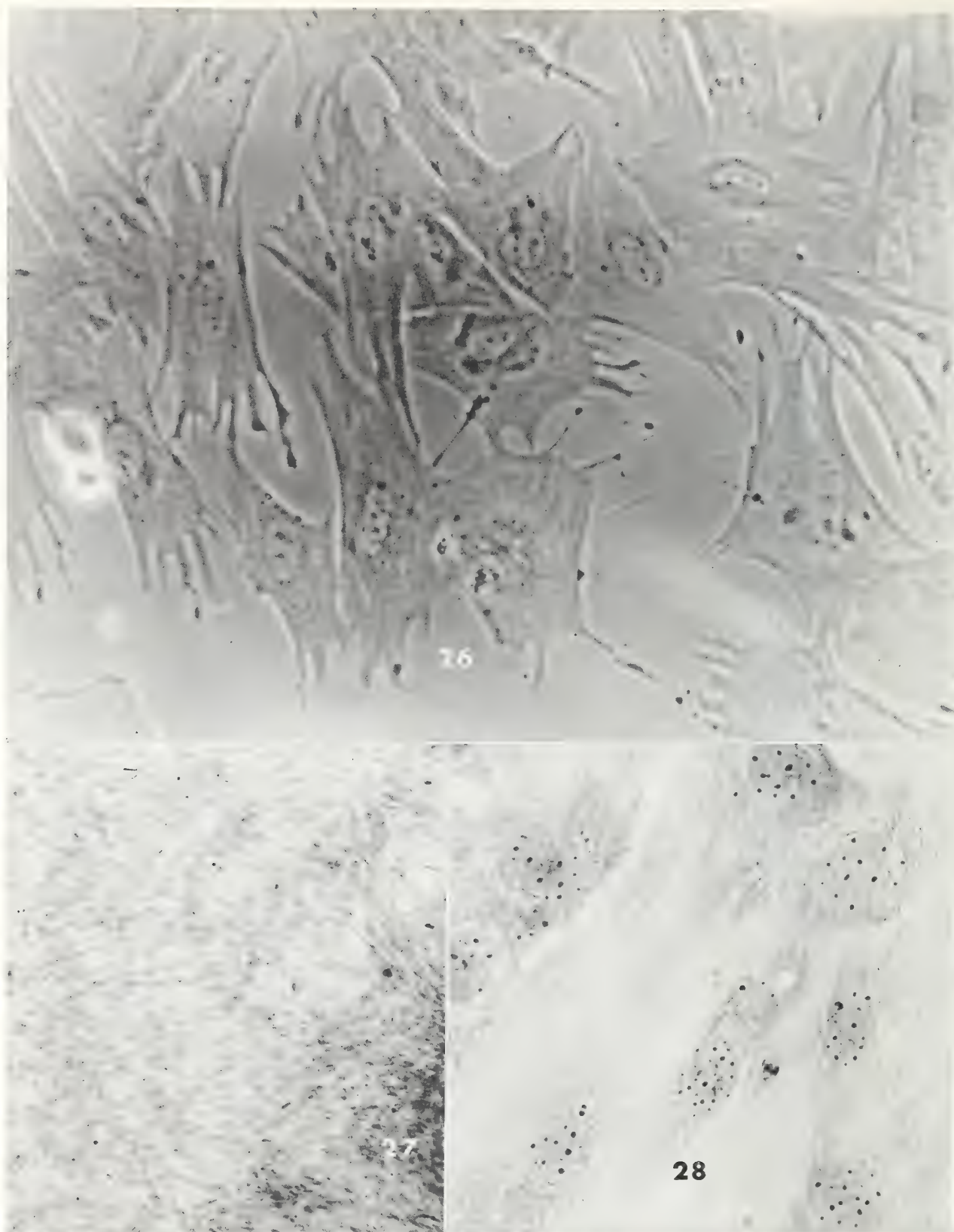


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FIGURES 20, 21, 22, and 23.



FIGURES 24 and 25.



FIGURES 26, 27, and 28.

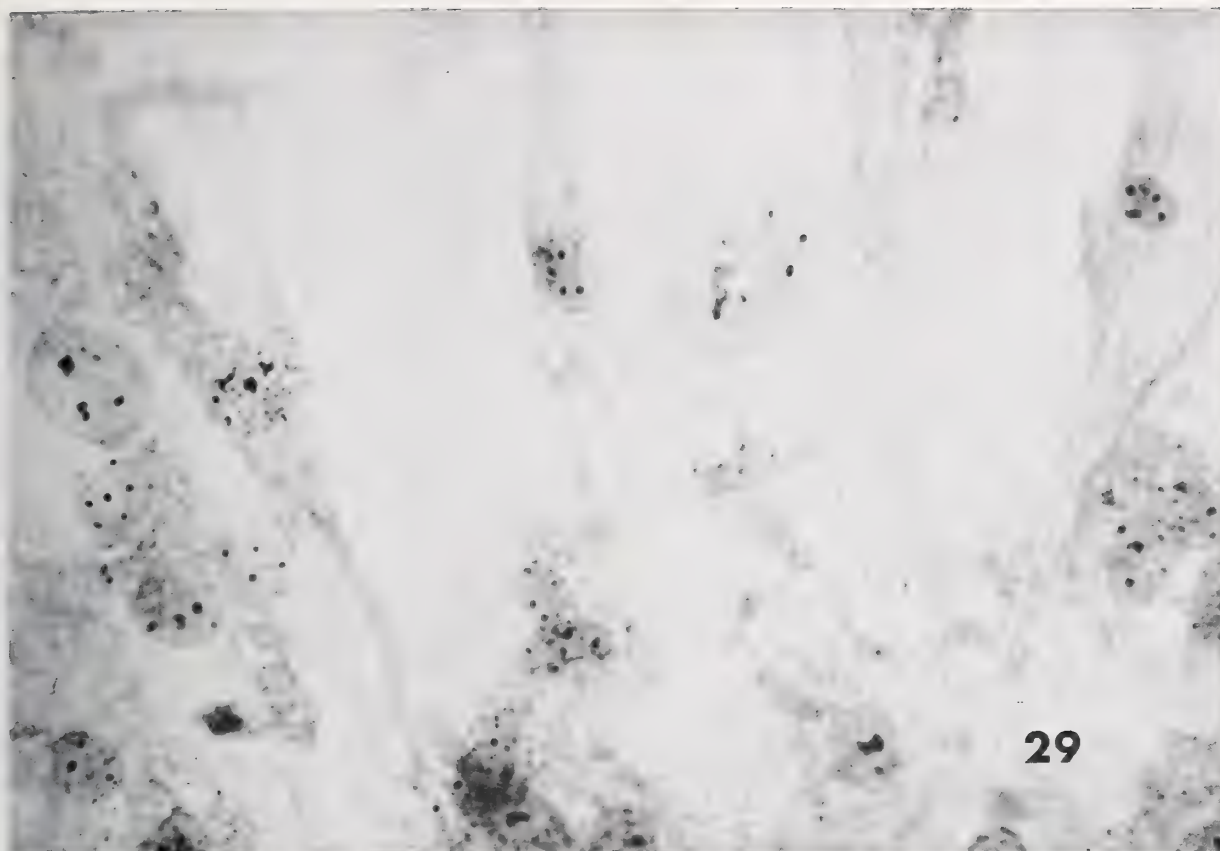


FIGURE 29.—Astrocytes in culture from the “normal” sheep brain exposed to fluids from “scrapie” sheep brain cell cultures for 33 days. The cultures were about 6 months in culture at the time medium transfers were initiated. $\times 640$. May-Grünwald Giemsa stain.

scrapie. Multinucleation is common; the range of nuclear size is wide; aggregations of cells with one or two nuclei having large areas of very thin cytoplasm are often found; and the tendency for varied sizes of astrocytes in random polarity to overlap or pile up in “pearl-like” aggregations is not uncommon. A large nucleus, sometimes with a twin, may be eight or more times as large as the largest nucleus to be found in astrocytes in cultures from mice not affected with the disease. Multinucleation occurs commonly in astrocytes of cultures from mice not affected with scrapie but noticeably less frequently than in similar cultures from mice affected with scrapie.

B. Inoculation of Mice with Cell Culture Medium

The results of the trials in which 13 groups of mice were each inoculated with different harvests of cell culture media are summarized in table I. It has been determined in previous trials that the two breeds of

mice used in these experiments were essentially equally susceptible. This observation was not materially altered by the results obtained thus far. The results in all trials were not complete at this writing. It is felt that useful information is available from incomplete trials and therefore has been included.

Five of the trials were complete at the time of this report. Two were the first groups of mice inoculated with cell culture medium from the primary culture derived from the sheep affected with scrapie. These were inoculated in November of 1962. Convincing signs of the disease were observed in 3 of 6 inoculated with inoculum No. 2 and these were killed 508 to 513 days post inoculation. All were males and over the period in which signs of the disease developed they were observed to show waddling gait, lethargy, rough soiled hair, priapism, plasticity of the tail, trembling, and finally somnolence. Histological preparations of the brain and thoracic portion of the spinal cord stained with Cajal's gold chloride sublimate method

TABLE I.—Tests for the Presence of Scrapie Virus in Cell Culture Fluids in Mice Exposed Intracranially

No.	Inoculum ¹	Mice	Scrapie	Elapsed time in months (trial completed)
1	P-SB-22D-F	5	1	(17)
2	P-SB-35D-F	6	3	(17)
3	SP23-SB-25M-7MEB	10	2	10
4	SP21,23,28-SB-27M	11	0	10
5	SP23-SB-14M	7	2	10
6	SP13-SB-10M	9	0	7
7	P-NSB+SM-43D	9	0	10
8	P-NSB+SM-40D	9	0	7
9	P-MB-10D	11	11	(9)
10	P-MB-30D-F	9	8	(15)
11	P-MB-38D	9	8	(6)
12	P-NMB-14D	11	0	10
13	P-NMB-14D	8	² 1	7

¹ See METHODS & Table of Abbreviations for details of inoculums.

² Focal astrocytic hypertrophy present in cord.

ABBREVIATIONS

SB=Sheep Brain cell culture-scrapie.
 NSB=Normal Sheep Brain cell culture.
 MB=Mouse Brain cell culture-scrapie.
 NMB=Normal Mouse Brain cell culture.
 MEB=days on Mouse Embryo Brain cell culture.
 P=Primary cell culture.
 SP=Serial Passage (followed by number).
 D=Days.
 M=Months.
 F=Filter 450 m μ .
 SM="Scrapied" Sheep cell culture Medium.

showed the astrocytosis and the extra-cellular vacuolation which attends scrapie in mice (1) and goats (12). The gray matter of sections of the spinal cord contained striking numbers of hypertrophic astrocytes especially in the ventral columns. In the second group inoculated at this time with inoculum No. 1, one of five White Swiss mice inoculated at 11 days of age was recognized to have developed scrapie. Others of the group may have, but were not recognized in this initial effort of lengthy duration. She was killed at 508 days showing signs typical of the disease which was supported by the presence of astrocytosis and extra-cellular vacuolation seen in histological preparations of the brain and spinal cord.

The three remaining trials which have been completed were observed more closely than the first two. In these trials mice were exposed to inoculums Nos. 9, 10, and 11. All of the mice so exposed developed scrapie except two that were lost from undetermined causes in the cage competition.

In the four trials in which inoculums Nos. 3, 4, 5, and 6 were used, and which were all harvested from cell cultures derived from the scrapied sheep, four mice had developed scrapie at the time of this report. Others have shown signs which are suggestive but were not convincing at the time.

In the two trials in which inoculums Nos. 7 and 8 were used no evidence of scrapie has been observed in the mice.

The groups inoculated with inoculums Nos. 12 and 13 were maintained in separate cages for 10 and 7 months at the time of this report and had shown no signs of scrapie. One from the group exposed to inoculum No. 13 was without changes indicative of scrapie on histological examination when killed at 4 months.

DISCUSSION

One of the concepts concerning the cells which seems to be crucial to this study is that the dominant cell type in cultures of the brain after about 2 weeks is the astrocyte. Nakai and also Lumsden have presented criteria for the identity of these cells (13, 14). The cells observed to constitute at least 95 percent of the cultures from both sheep and mice whether normal or affected with scrapie seemed to fulfill the criteria for astrocytes. Therefore, our best estimate of their identity was so presented in the description of the results of observations of the cultures.

Hope for the presence of scrapie virus in the cell cultures of "scrapied" sheep brain arose initially by observations of differences between the character of the colonies and the morphology of the astrocytes when compared to similar cultures from a sheep not affected with scrapie. In general the differences were not as clear cut as those which might be anticipated in cytopathic effects of temperate virus infections, but were relative. The differences were sometimes striking but were always relative and seemed to be reminiscent of neoplasia. The greater range of cellular and nuclear size, greater order and index of multinucleation, greater tendency to pile up or overlap, greater tendency for granularity and the lack of polarity which often attended the scrapie cell cultures characterized the morphological differences. There seemed to be a propensity for exaggeration of these characteristics on the part of the astrocytes from the sheep with scrapie. Such impressions were developed through observations of the cell cultures through 30 months and a maximum of 34 serial subcultures of the cell cultures from the sheep affected with scrapie. Even so one must keep in mind that all the cultures examined and watched

through this period came from one sheep with scrapie and were compared with similar cultures from one sheep which did not have scrapie. However, similar differences, presented in RESULTS, were observed between cultures from mice affected with scrapie and those not affected, in several trials of 60 days or less. The relationship of these differences in cell culture characteristics to the presence of scrapie has yet to be established definitively. This is directly related to the fact that there is no serological test for identification of the virus. The inoculation trials of cell culture fluids harvested from the scrapie source cultures are incomplete. However, scrapie has developed in mice inoculated with materials from four different harvests by this time and not in mice exposed to similar material from cell cultures derived from the sheep not affected with scrapie. The clinicopathological requirements for the diagnosis of scrapie in mice were fulfilled for those exposed to cell culture fluids from the 23d serial subculture after 14½ and 25 months in culture as well as fluids from primary cultures at 22 and 35 days of culture. Evidence of the presence of scrapie virus in cell cultures of the brain of a sheep affected with scrapie has been obtained.

The presence of scrapie in cell culture preparations from the brains of mice affected with scrapie from the Compton source has been demonstrated by inoculation of mice in similar fashion. The fluids used in the tests in mice were harvested from primary cell cultures. The critical consideration is whether or not the virus demonstrated by inoculation of mice represented dilution of residual virus or that which was propagated in the cells during the culture. At the initiation of the culture the cells and attendant debris are washed in BSS and dispersed in complete medium. Virus associated with the debris and cells which becomes free in the medium is available for dilution during the medium changes prior to harvest. The first change approximately halves the volume. Subsequent changes were as complete as is possible by pouring off all that will pour. For the sake of discussion it is assumed that ten-fold dilutions are accomplished by this technique. Therefore, the approximate dilution achieved with inoculum No. 9 was 2×10^{-2} ; with inoculum No. 10 it was 2×10^{-8} ; with inoculum No. 11 it was 2×10^{-10} . These dilutions are not very impressive, nevertheless when coupled with the morphological changes observed in the astrocytes of the cell cultures it provides reason to continue the effort. While it has not been difficult to initiate mouse brain

cell cultures we had not tried to subculture them during the period covered by this report. The longest period of maintaining the cultures has been 68 days.

SUMMARY

Monolayer cell cultures from the brain of a sheep affected with scrapie virus isolated in Indiana were grown for 30 months during which as many as 34 serial subcultures were made. Scrapie has developed in some mice exposed to filtrates of fluids harvested at various times from the cultures. The disease developed in mice inoculated with fluids harvested after as many as 23 serial subcultures of the cells. When compared with similar cultures from a sheep not affected with the disease, the astrocytes from the affected sheep in culture had a wider range of whole cell and nuclear size, a greater order and intensity of multinucleation, a greater propensity for overlapping and "piling up," a lack of colonial polarity, often a greater intensity of cytoplasmic granularity, and more frequent presence of small satellite and bizarre nuclei.

Monolayer cell cultures derived from whole brains of "scrapied" mice exposed to the Compton strain of scrapie virus differed from similar cultures from normal mice in much the same fashion as in the cultures from the sheep. Mice inoculated with fluids harvested from the cultures up to 38 days of culture developed scrapie.

Several of the trials were incomplete at the time of this report although some of the principals contributed to the evidence of the presence of scrapie virus in cell culture fluids.

ACKNOWLEDGMENT

The strain of scrapie virus from the Agricultural Research Council Field Station, Compton, Berkshire, England, was obtained through the courtesy of Dr. Wm. J. Hadlow of the USPHS, NIAID, Rocky Mountain Laboratory, Hamilton, Mont.

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DISCUSSION

BECK: I wonder if you examined sections of the cord for vacuolation in anterior horn cells. This, we have found, is perhaps even more specific than the astrocytes. In most of the scrapie mice we found the anterior horn cells severely vacuolated and there was even loss of anterior horn cells; the motor nuclei in the brainstem were also affected. I suggest that you look in the seventh nerve nucleus, which is very large in mice; in some of the mice we examined practically every cell showed typical vacuolation.

HADLOW: I would like to reiterate what was mentioned earlier that when we talk about scrapie we are talking about a clinico-pathologic entity. That is the only way we can put a label on it. So, in the end, whether we accomplish the induction of scrapie depends upon whether we obtain what we have accepted as the typical clinical picture, and then confirm this by finding the typical pathologic changes. Undoubtedly, I have influenced Dr. Gustafson in the use of the astrocytes because, as far as the lesions are concerned, the earliest unequivocal change we can see is in the astrocytes. Astrocytes are difficult to impregnate in the normal mouse, but, seemingly, once the onset of the disease occurs, coincident changes take place in astrocytes which make them very readily impregnated. So, in the end, the criteria we have used have been a typical clinical picture, which is monotonously clear-cut because it appears all the time in the same way, and this astrocytic response.

Q: While one is looking for a way of judging the appearance of clinical disease, one index is available in mice that might be worth trying, and that is electroencephalography. Intracortical recording is a relatively sensitive thing and has been done in mice, and it might be worthwhile to take a group of scrapie mice and compare them with normals and see if it is a useful clinical index.

FIELD: We happen to have an M.R.C. Psychiatric Unit nearby, so I gave them some scrapie mice to test. They have discovered that these animals react abnormally, and they react abnormally about 6 weeks after they have been inoculated intracerebrally, at a time when you can just begin to see a few astrocytes appearing more prominent than normal; and it is 2 or 3 months before we get definite clinical signs, so that, even in the long incubation period, there is an abnormal behavioral pattern which we can bring out by psychometric testing.

Observations on the Experimental Transmission of Scrapie of Various Origins to Laboratory Animals

I. ZLOTNIK

Moredun Institute, Gilmerton, Edinburgh, Scotland

Scrapie has always been considered to be a disease that affects only sheep under natural field conditions. Cases of scrapie in goats are thought to be very rare and to occur only under exceptional circumstances (Chelle (1), Mackay and Smith (2), and Stamp (3)). Many breeds of sheep are known to have had scrapie: Cheviot, Suffolks, Border-Leicester, Swaledales, Dalesbred, Wensleydales, Southdowns, Dorsetdowns, and Welsh mountain. Although various forms and clinical syndromes have been described in the sheep, the general opinion was that the disease is homogeneous and that the clinical picture depends, to a large extent, on the location of the brain lesions. Such an opinion was justified in view of the results of histological examinations of the central nervous system, in the absence of any detectable immunological reaction, and because during many years the Cheviot was the only animal used for the study of the transmission of the disease, a breed of sheep where experimentally transmitted scrapie produces a characteristic clinical picture and a rather uniform pattern of brain lesions.

In 1939, Cuillé and Chelle (4) reported the successful transmission of scrapie to goats, but the disease in that animal remained almost unknown to the majority of research workers until Pattison et al. (5) demonstrated the high degree of susceptibility of the goat to scrapie and began using goats in increasing numbers for the study of scrapie. Later, Pattison and Millson (6) reported the occurrence of two clinical syndromes in the experimentally infected goat, which they described as nervous or drowsy and scratching. These syndromes appeared to be reproducible by subsequent intracerebral inoculations. The latter publication constitutes the first suggestion that scrapie might not be

a homogeneous disease after all, in spite of the fact that the differentiation of goat scrapie into two types was based on clinical and experimental evidence, without however the possibility of demonstrating any difference in the histological brain lesions (Zlotnik (7)). This is of special interest in view of finding histological differences between the distribution and the severity of the characteristic changes in the brain of scrapie-affected sheep of the Cheviot and Suffolk breeds and that no great difference could be found between lesions of naturally and experimentally affected sheep of the same breed (Zlotnik (8)).

Further evidence that differences may be present between scrapie from sheep of various breeds or between goats affected with the two syndromes is supplied by the results of transmission experiments of scrapie to laboratory animals (Chandler (9, 10, 11), Chandler and Fisher (12) and Zlotnik and Rennie (13, 14, 15)). In the present survey details of transmission experiments are given, which show that animals inoculated with scrapie material from various sources may exhibit great differences in their susceptibility to the disease in the length of the incubation periods and in the type and distribution of brain lesions. All these results, although not constituting conclusive proof of the existence of scrapie strains in the strict sense show that reproducible variants of scrapie, depending on the origin of the inoculum, can be distinguished by means of experimental transmission and histological examinations.

MATERIAL AND METHODS

The following animals were used in the present study: sheep, goats, rats, hamsters, and mice. The

sheep were of the Cheviot and Suffolk breeds and of various ages from 5 months to 3 years old. All the goats were castrated males of various breeds about 4 to 5 months old. The laboratory animals consisted of Hooded Lister rats 5 weeks old, Golden hamsters 6 weeks old and Swiss White mice between 3 and 4 weeks old.

The inocula consisted of supernates of 10 percent homogenates of brain or spleen in saline after 15 minutes centrifugation at 2,000 g. Sheep and goats received either 1.0 ml. intracerebrally or 5.0 ml. subcutaneously, rats and hamsters 0.1 ml. intracerebrally or 1.0 ml. intraperitoneally, and mice were given 0.03 ml. intracerebrally or 0.03 ml. intraperitoneally.

In all experiments the brains of the inoculated animals were removed immediately after destruction by complete exsanguination and, while parts were stored at -20° C. for subinoculations, at least half brains were fixed in 10 percent formol saline for 2 to 4 weeks. The techniques of fixation and preparation of brain sections for histological studies were similar to those described elsewhere (Zlotnik and Rennie (13)).

RESULTS

I. The Transmission of Scrapie from Sheep

Attempts have been made to reproduce scrapie in laboratory animals and although many failed a number of successful transmissions have been carried out. Brain and spleen tissue from scrapie-affected sheep of many breeds have been used as inocula in transmission experiments in mice, and various routes of inoculations were employed, such as the intragastric, intracerebral, subcutaneous and intraperitoneal. Hitherto only inoculations of mice with tissues from Suffolk sheep and from one case in a Southdown proved successful, while attempts to transmit scrapie to mice from Cheviot sheep, affected either naturally or experimentally with scrapie, from Swaledales and half-bred sheep did not give positive results. Hamsters and rats were successfully infected with mouse-passaged scrapie, while rabbits and guinea pigs remained refractive to both sheep tissues and mouse tissues.

A. The Transmission of Scrapie from Suffolk Sheep (Figure 1)

A total of eight separate experiments have been carried out in the last 4 years, involving inoculations of mice with material from scrapie-affected Suffolk sheep and all proved successful. Although all these transmissions have been maintained in further mouse

passages the bulk of experimental work was centered around one large scale experiment where a great number of sheep, goats, hamsters, rats, and mice were used. The results of this experiment proved that scrapie can be transmitted to mice from sheep and that mouse-passaged scrapie will produce the disease when inoculated into hamsters, rats, goats, and sheep (Zlotnik and Rennie (15)). The experiment started over 4 years ago when brain and spleen pools from naturally affected Suffolk sheep were given by the intragastric route to mice and brain material of the same origin was inoculated simultaneously by both the intracerebral and the subcutaneous routes into Suffolk sheep.

(a) The Transmission of Scrapie from Mice Inoculated by the Intragastric Route

Thirteen months following the intragastric inoculations of mice with either brain or spleen from Suffolk sheep, no symptoms could be observed in test animals, but on histological examination, brain lesions were found, consisting of neuronal degeneration and typical vacuolation accompanied by status spongiosus in subcortical centers along the neuraxis. Brain and spleen pools made from the above subclinical cases were subsequently inoculated into further mice by the intracerebral (1) or intraperitoneal routes (2).

1. The Intracerebral Route

Four groups of mice were inoculated by the intracerebral route and within 5 to 6½ months all mice developed symptoms. Mice that were not destroyed during the course of the disease died and the actual period from the onset of symptoms till natural death varied from a few days to several weeks. Three definite syndromes were recognized, the hyperexcitable, the fat, and the lethargic. The hyperexcitable syndrome had a rapid course, the fat type was usually chronic and protracted and the lethargic type, which forms by far the most common syndrome of the disease, usually lasted a few weeks. Animals irrespective of the syndrome had similar brain lesions in the form of widespread status spongiosus, degeneration and vacuolation of neurones and hypertrophy of astrocytes, but unlike first passage mice the lesions were present throughout the brain including the cerebral cortex and hippocampus (figs. 2 and 3). A feature of special significance is the vacuolation in the lateral vestibular nuclei of the medulla-pons, a site invariably affected in all mice inoculated with mouse-passaged Suffolk sheep scrapie. Brain material from mice of the second passage was inoculated into further mice and into rats.

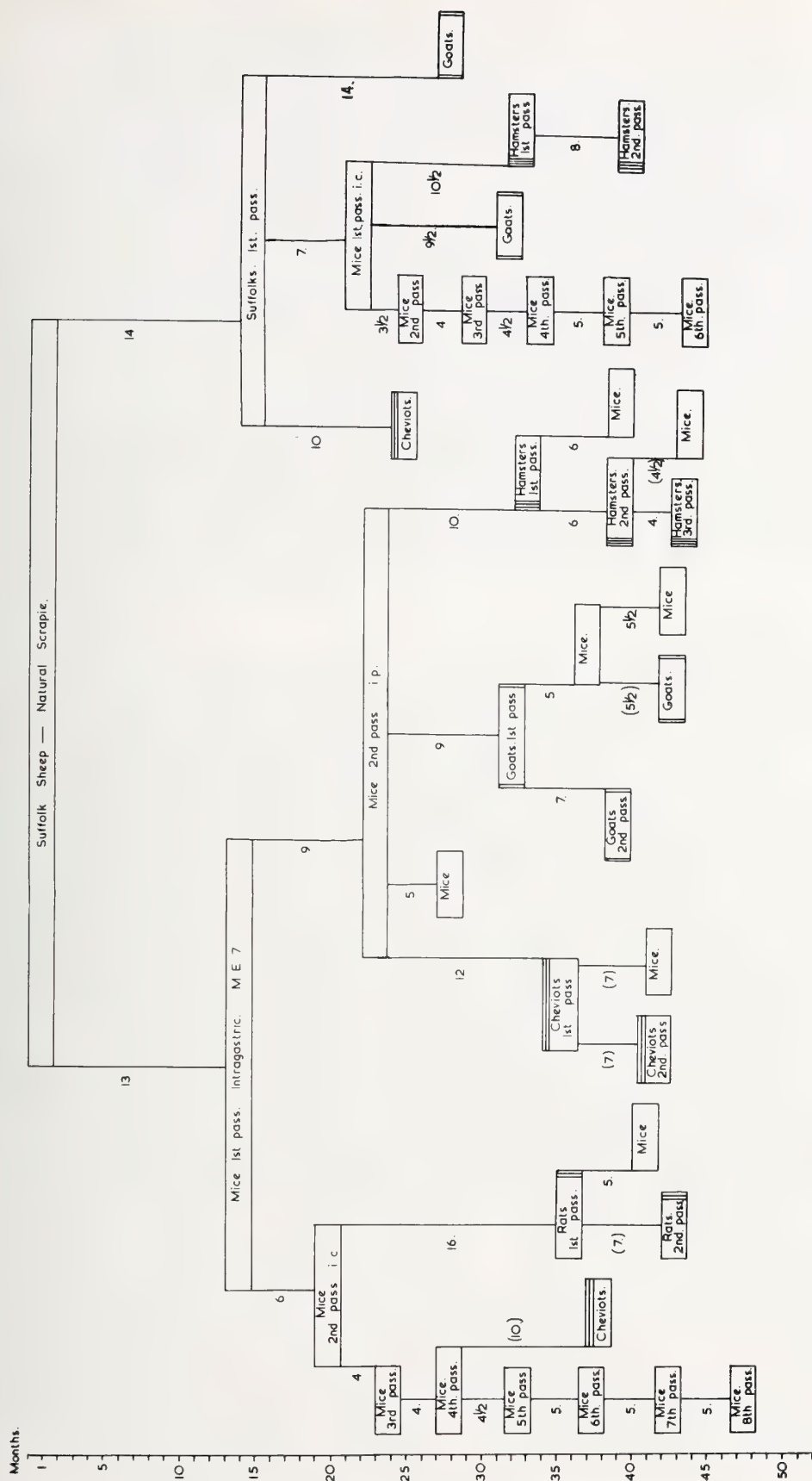


FIGURE 1.—The transmission of scrapie from Suffolk sheep. Figures denote incubation period in months. Figures in parenthesis show number of months from the date of inoculation in which no cases of scrapie occurred.

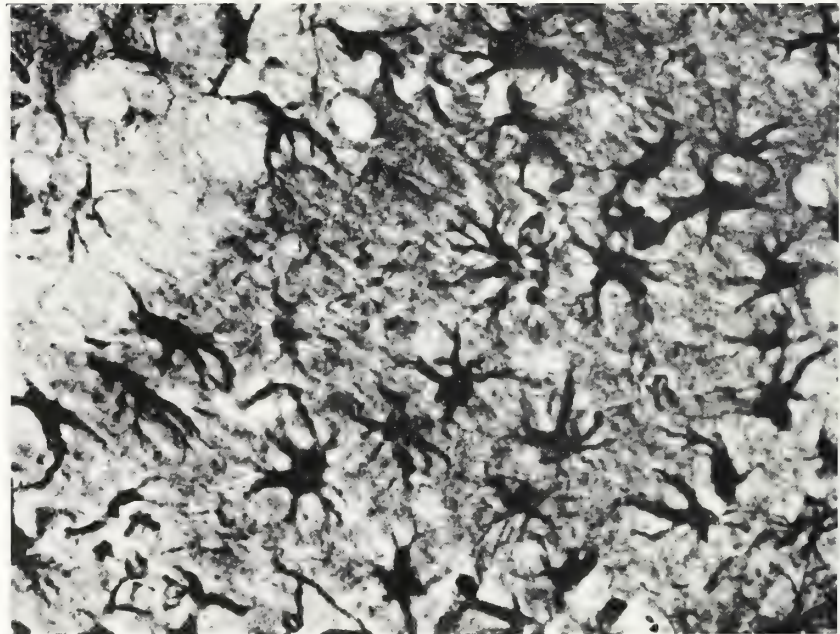
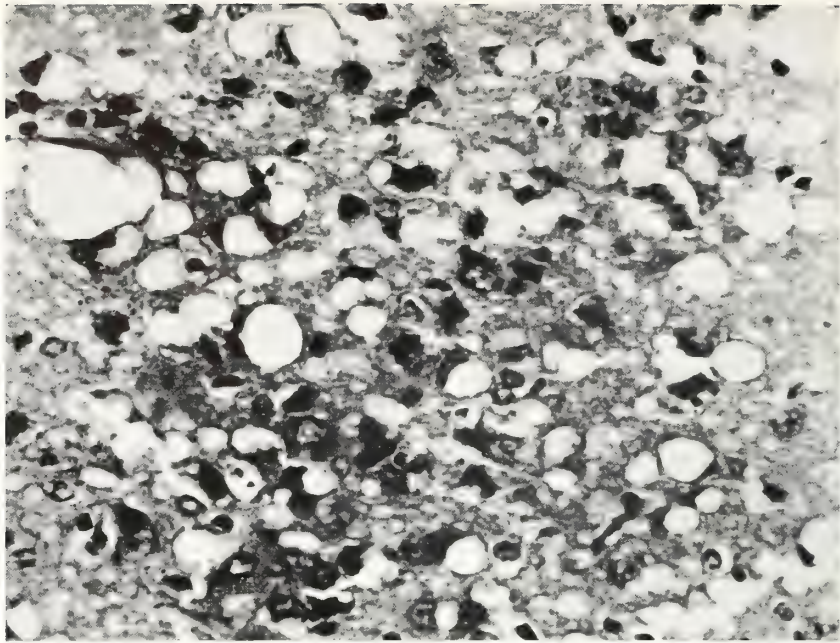


FIGURE 2.—TOP—Status spongiosus of the hippocampus of a mouse affected with scrapie. H. & E. $\times 420$

FIGURE 3.—BOTTOM—Swelling of astrocytes in the hippocampus of a mouse affected with scrapie. Cajal. $\times 420$

A total of eight passages in mice have now been carried out and in all passages from third onward the incubation period was reduced to between 4 and 5 months. The clinical picture and brain lesions were similar to those in mice of second passage.

Rats inoculated with mouse brain of the second mouse passage developed clinical symptoms after 16 months. The affected rats lost body condition, salivated excessively and when made to move the hind legs were limp and were dragged forwards by the movement of the forelegs and body. Very severe lesions were found in the brains of affected rats, which were similar to those present in the brain of mice of the second mouse passage and were widely distributed throughout the brain including the cerebral cortex, hippocampus and the lateral vestibular nucleus. Brain from the above clinically affected rats was subinoculated into further rats and mice. While no symptoms were observed in the rats within 7 months from their inoculation, all mice that received the same rat brain suspensions developed symptoms and typical brain lesions after an incubation period of only 5 months (Zlotnik and Rennie (15)).

2. *The Intraperitoneal Route*

Two groups of mice that received intraperitoneal inoculations of mouse brain and spleen of first sub-clinical mouse passage (intragastric route), developed symptoms after an incubation period of 9 to 9½ months. The clinical picture and the brain lesions in these mice resembled those of the intracerebral second passage in mice. Brain material from the above mice infected intraperitoneally was subinoculated into four different groups of animals as follows: mice, goats, hamsters, and Cheviot sheep. Five months after inoculation all mice that received mouse brain of second intraperitoneal passage in mice developed symptoms and brain lesions. Four months later, after an incubation period of 9 months, all four inoculated goats became affected with the scratching syndrome of scrapie. Brain lesions were found in all brains and these were similar in all respects to goat scrapie except that lesions were not confined to subcortical centres but were also present in the cerebral cortex, hippocampus, amygdaloid nucleus, and corpus striatum (Hadlow (16), Zlotnik (7, 8, 17), and Zlotnik and Rennie (15)). Subinoculation of goat brain into further goats and mice produced scrapie after 5 months in mice and after an incubation period of 7 months in goats (2d passage). The brain lesions of the goats were similar to those of first passage of mouse-passaged scrapie in goats while the lesions in the mouse

brain were the same as in mice inoculated with mouse-passaged scrapie. It is worth noting that a further sub-inoculation of mice with brain from the above mice resulted in scrapie in 5½ months (Zlotnik and Rennie (15)). Ten months following the inoculation of hamsters with mouse brain of the second (intraperitoneal) passage, symptoms appeared which consisted of drowsiness, loss of natural aggressiveness, unsteady gait, incoordination, complete prostration, and death after a period of coma. The brain lesions in these hamsters resembled in all respects those of mice and rats, they were very widespread and affected all parts of the brain (Zlotnik (18)). Subinoculation of brain material into further hamsters and mice gave rise to symptoms in both mice and hamsters after an incubation period of 6 months. In the third hamster to hamster passage, using brain as inoculum, the incubation period was again reduced to only 4 months.

Of the 25 Cheviot sheep that received subcutaneous inoculations of mouse brain of second (intraperitoneal) mouse passage, only two animals developed classical symptoms after an incubation period of 12 months. Brain and spinal cord lesions were very widespread in the two affected sheep and consisted of both neuronal vacuolation and degeneration as well as hypertrophy of astrocytes and status spongiosus in most areas of the brain (figs. 4 and 5). Thus very severe changes were present in the cervical cord, in the medulla, including the inferior olives and the cuneate nuclei, pons, mid-brain, cerebellum, thalamus, hypothalamus, and focal changes were also seen in the hippocampus, cerebral cortex, and paraterminal body (Zlotnik and Rennie (15)). It appears therefore that lesions in these sheep were more severe and of a different pattern than those usually present in experimental scrapie of sheep (Zlotnik (8, 19, 20)).

(b) *The Transmission of Scrapie from an Experimental Suffolk Sheep*

Fourteen months following the combined intracerebral and subcutaneous inoculations of Suffolk sheep with brain pool from naturally affected Suffolk sheep, one animal developed typical symptoms of scrapie. The disease in that sheep progressed very rapidly and within 1 month the animal had to be destroyed. Brain suspensions from the above sheep were inoculated into 4 goats, 20 Cheviot sheep and 40 mice. Seven cases of scrapie were recorded in subcutaneously inoculated sheep after an incubation period of 10 to 12 months; mice that received intracranial inoculations developed scrapie in 7 months while subcutaneously

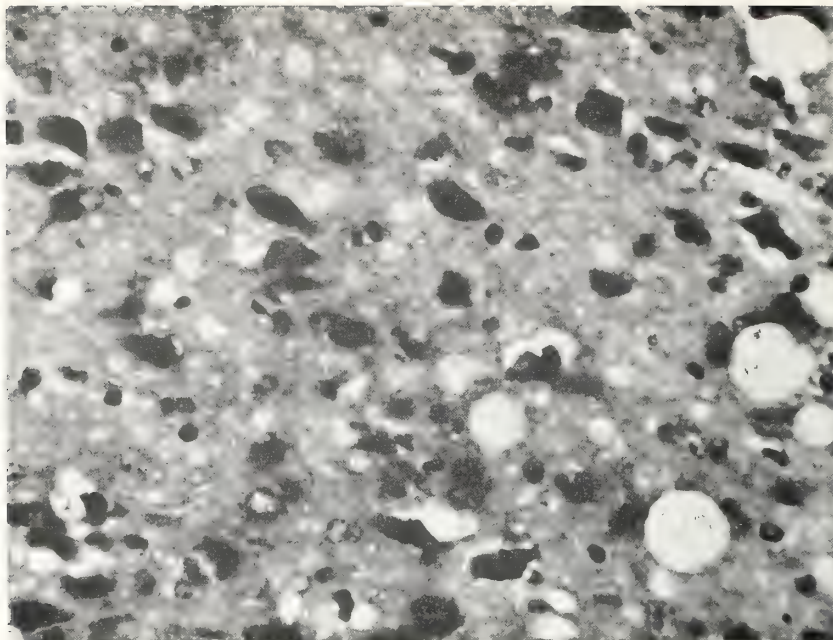


FIGURE 4.—TOP—Status spongiosus in the gyrus hippocampi of Cheviot sheep inoculated with mouse-passage scrapie. H. & E. $\times 420$.

FIGURE 5.—BOTTOM—Swelling of astrocytes in the gyrus hippocampi of Cheviot sheep inoculated with mouse-passaged scrapie. Cajal. $\times 420$

inoculated mice did not start showing symptoms till a period of 10 months elapsed (Zlotnik and Rennie (14)). All mice inoculated with Suffolk brain by the intracerebral route had brain lesions confined only to the subcortical centers along the neuraxis. The four goats that were inoculated with the same Suffolk brain developed symptoms 14 months following the intracerebral inoculations. Mouse brain suspensions of the above first mouse passage (intracerebral) were further subinoculated into mice, goats, and hamsters.

The incubation period of mice of second passage was dramatically reduced to 3½ months but on further subinoculations it varied from 4 to 5 months. Six intracerebral passages have now been carried out and symptoms and lesions in all the mice resembled those of mice of second and further passages inoculated with mouse-passaged scrapie that originated from the intragastric transmission (see under (a)). Brain lesions were as a rule very severe, widespread, and affected all parts of the brain, including cerebral cortex. Neuronal vacuolation in the medulla, pons and mesencephalon was invariably present along with other forms of neuronal degeneration, swelling of astrocytes and status spongiosus.

All four goats inoculated with mouse brain of the above first mouse passage developed symptoms of scrapie within 9½ months. Histological examination of the brain of these goats revealed widespread lesions which were, however, confined to the subcortical centers along the neuraxis, including vacuolation of the cuneate nuclei of the medulla.

Ten and a half months following intracerebral inoculations all hamsters developed scrapie and on further subinoculation of hamster brain into hamsters the incubation period was reduced to 8 months. Clinical symptoms and brain lesions in both first and second hamster passage resembled those described above for hamsters inoculated with mouse brain that originated from the intragastric transmission.

B. The Transmission of Scrapie from a Southdown Ewe

Brain from a Southdown ewe that developed natural scrapie, when inoculated into mice produced symptoms after 11 months. Subinoculation of mouse brain into further mice caused a shortening of the incubation to 8 months. Symptoms and brain lesions of first passage mice resembled those of mice inoculated with experimental Suffolk sheep brain; however, in mice of second mouse to mouse passage brain lesions were found only in the medulla, pons, mesencephalon and hypo-

thalamus without affecting the cerebral cortex or hippocampus.

II. The Transmission of Scrapie from Goats (Figure 6)

The first successful transmission of scrapie from goats to mice was carried out in the Moredun Institute by means of inoculations of mice with brain suspensions from goats affected experimentally with the scratching syndrome. Later brain from an uninoculated goat showing the scratching syndrome was also inoculated in mice (Mackay and Smith (2)). Finally, in order to compare the clinical symptoms and brain lesions of mice inoculated with scratching goat scrapie with those inoculated with drowsy goat scrapie (Chandler (9, 10, 11), and Pattison and Smith (21)) material from a brain pool of drowsy goat scrapie was also passaged through mice.

(a) Transmission of Experimental Scrapie of Goats—Scratching Syndrome

Suspensions from pooled brains of two goats were inoculated intracerebrally into goats and mice. Similar suspensions from brains of the same goats were inoculated into 30 Cheviot sheep, and resulted in 2 cases of scrapie, after an incubation period of 12 months (Mackay et al. (22)). Inoculated goats developed scrapie within 8 to 9 months while mice started to show definite symptoms after 12 months. Subinoculation of brain material from first passage mice resulted in a drastic reduction of the incubation period to 4½ months in second passage and 3½ months in third, but in subsequent fourth to sixth passages the incubation period remained static at 5 months. Clinical symptoms in inoculated mice did not differ from those inoculated with mouse-passaged Suffolk scrapie. Similarly, brain lesions of first mouse passage consisted of degeneration and vacuolation of neurones, swelling of astrocytes and status spongiosus of subcortical centres. The typical vacuolation of neurones in the lateral vestibular nuclei was invariably present in all examined brains. On second and further passages, however, brain lesions extended to all parts of the brain, especially the cerebral cortex, hippocampus, corpus striatum, and thalamus.

Mouse brain of second mouse passage was inoculated into goats, hamsters, and rats in addition to the above mentioned mice. All inoculated goats and hamsters developed scrapie within 9 months but no symptoms were observed in rats during the 17 months they remained under observation. After 17 months

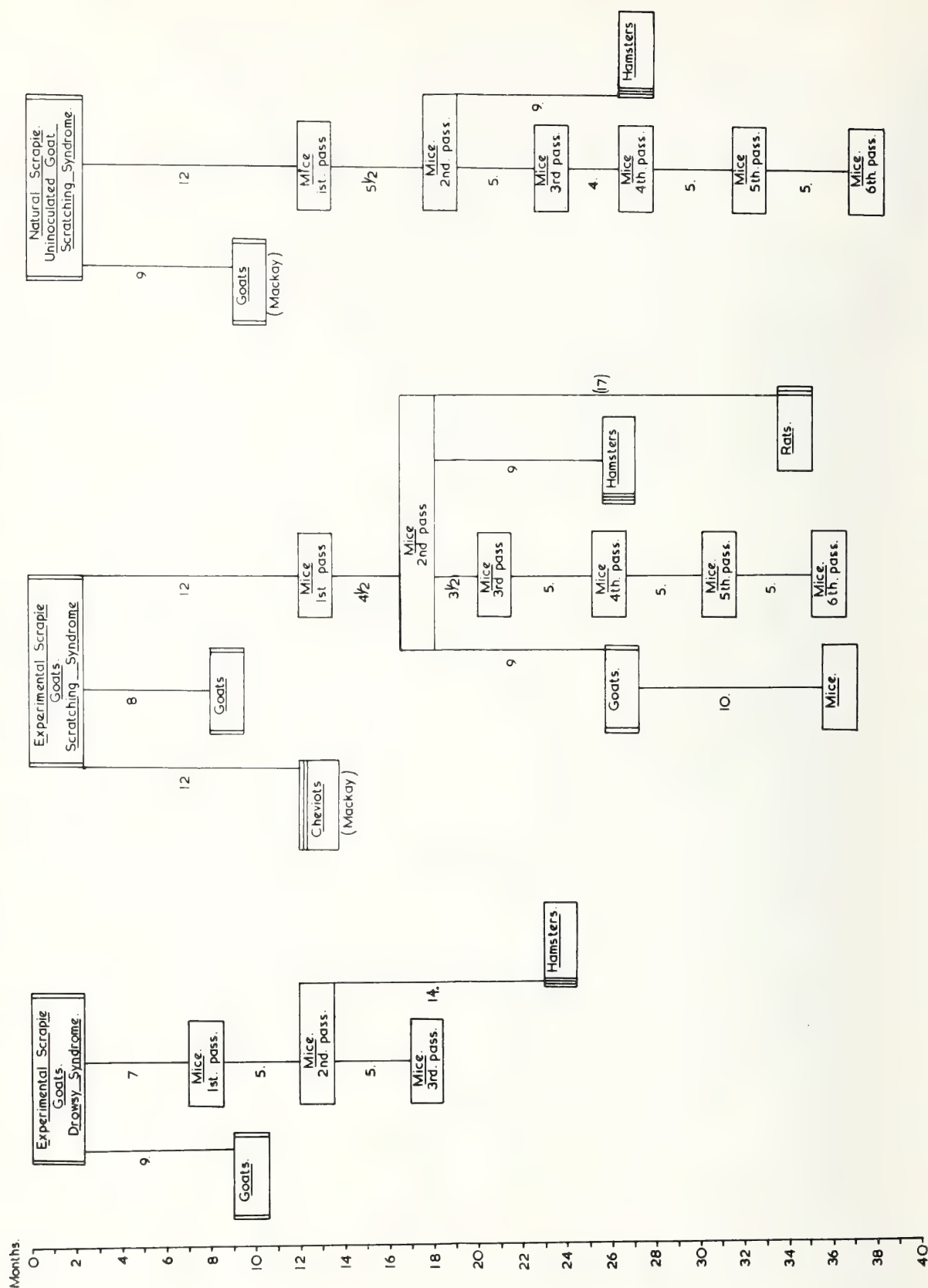


FIGURE 6.—The transmission of scrapie from goats. Figures denote incubation period in months. Figures in parenthesis show the number of months from the date of inoculation in which no cases of scrapie occurred.

the rats were destroyed but no lesions could be found in their brains.

The clinical picture and brain lesions of hamsters and goats inoculated with mouse-passaged scratching-type goat scrapie were similar in all respects to those inoculated with mouse-passaged scrapie of Suffolk sheep origin. On the other hand when brain material of the above scrapie goats was inoculated into mice the incubation period was extended to 10 months, but the clinical symptoms and the brain lesions of these mice were exactly the same as those in mice of second mouse passage.

(b) Transmission of Experimental Scrapie of Goats—Drowsy or Nervous Syndrome

Suspensions of a brain pool of goats affected with the drowsy syndrome when inoculated into goats and mice produced symptoms in goats after 9 months, while the mice developed scrapie within 7 months. Although clinical symptoms resembled in many respects mice inoculated with the scratching syndrome, the brain lesions consisted only of status spongiosus and hypertrophy of astrocytes of the subcortical centres and the cerebellum. Subinoculation of brain material from these mice into further mice produced symptoms in 5 months and brain lesions extended to all parts of the brain including cortex, thalamus, corpus striatum, and hippocampus. Although the main lesion consisted of status spongiosus and astrocytic swelling, cortical neurones were often vacuolated, but in no case, however, were the neurones of the lateral vestibular nuclei showing the typical simple and multiple vacuoles which are usually present in mice inoculated with mouse-passaged scratching scrapie. Inoculation of brain material from second passage mice into mice and hamsters, resulted in scrapie in the third passage of mice after 5 months' incubation, but no symptoms could be observed in the hamsters till 14 months following inoculation.

(c) Transmission of Natural Scrapie of Goats—Scratching Syndrome

Brain material from an uninoculated goat affected with the scratching syndrome of scrapie was inoculated into a group of mice. At the same time (Mackay, unpublished personal communication) a similar suspension of the above goat was inoculated into further goats and resulted in a very severe scratching syndrome in all the inoculated goats after an incubation period of 9 to 12 months.

Twelve months following intracerebral inoculations the mice started showing very indefinite symptoms, and histological examination of brains of these mice re-

vealed very characteristic neuronal lesions in the medulla and pons including severe vacuolation of the lateral vestibular nucleus and status spongiosus in the mesencephalon. Subinoculation of brain material from these mice into further groups of mice resulted in clinical symptoms within 5½ months and brain lesions extending to all parts of the brain including the cortex and the hippocampus. Six passages have now been carried out and the incubation period as a whole remained approximately at 5 months.

Mouse brain of the above second mouse passage was inoculated into hamsters and resulted in symptoms of scrapie within 9 months. Both symptoms and brain lesions of the above hamsters were similar to those inoculated with mouse-passaged experimental scratching scrapie of goats.

DISCUSSION

The results of the present work show that tissues from scrapie sheep and goats of various origins are capable of transmitting scrapie to mice. Mouse-passaged scrapie can be further transmitted to rats, mice and hamsters or back to sheep and goats. The successful passage of scrapie to Cheviot sheep and goats from second passage mouse material, the passages in these mice having been made in the first instant by the intragastric route and then by the intraperitoneal route, presents proof of true transmissibility of scrapie to mice.

The transmission of scrapie to mice seemed to have had a profound effect on the scrapie agent in that the mouse-passaged scrapie when transmitted back to sheep and goats produced characteristic brain lesions, not only in subcortical centers along the neuraxis, but also in the cerebral cortex, amygdaloid nucleus, and hippocampus. Further, when mouse-passaged scrapie is transmitted from goats, rats, and hamsters back to mice the disease in these mice and the cerebral lesions remain the same as in mouse-passaged scrapie, as if the disease has been passed directly from mouse to mouse. Similarly, brain of goats that developed scrapie as a result of inoculation with mouse-passaged scrapie when inoculated into other goats gave rise to brain lesions similar to those produced by inoculation with mouse brain. Additional evidence of the change in the scrapie agent caused by passing through mice may be seen by comparing the result of inoculation of experimental scrapie Suffolk sheep brain directly into goats with that of brain passage once through mice: the same Suffolk brain when passaged once through

mice caused scrapie within 9½ months in all inoculated goats while direct inoculation of Suffolk brain gave positive results only after 14 months.

Passaging through mice and other laboratory animals may also provide evidence of differences between scrapie of various origins. Thus, inoculations of mice with tissues from scrapie sheep and goats gave rise to scrapie in mice as follows: 7 months after inoculation with brain from goats affected with the drowsy syndrome and experimentally affected Suffolk sheep, 11 months following inoculation with the Southdown brain, 12 months with goat brain of the scratching scrapie syndrome and between 13 to 15 months with naturally affected Suffolk sheep brain, while transmission to mice of Cheviot, Swaledale, and halfbred brains proved hitherto unsuccessful.

When goat brain of the drowsy and scratching syndromes was inoculated into mice, not only were differences found in the lengths of the incubation periods, but also in the type of brain lesions and in the relative susceptibility of hamsters to inoculations with mouse brain of the two passages. At the same time the passage through mice was almost identical when scratching experimental scrapie goat brain and the brain from the naturally affected goat was inoculated into mice.

Finally, when mouse brain of the second mouse passage of Suffolk sheep and scratching goat scrapie was inoculated into goats the incubation period, irrespective of the inoculum used, was 9 months, but when brain of these goats was subinoculated into mice the incubation period was 5 months after inoculations with goat brain of mouse-passaged Suffolk sheep scrapie and 10 months after mouse-passaged goat scrapie.

SUMMARY

The direct transmission of scrapie to mice from Suffolk and Southdown sheep and from goats affected with either the scratching or drowsy syndromes have been discussed.

The differences in the length of the incubation periods and in the distribution of brain lesions in inoculated mice points to the existence of more than one type of scrapie.

Mouse-passaged scrapie when inoculated into other animals is capable of reproducing the disease in sheep, goats, hamsters and rats. The passage of scrapie through mice has caused a change in the agent in view of the fact that animals inoculated with scrapie mouse brain or spleen of the second or further passages

will develop lesions not only in the subcortical centres along the neuraxis but also in the cerebral cortex, hippocampus, and the amygdaloid nucleus.

ACKNOWLEDGMENTS

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DISCUSSION

POSKANZER: What structures do the vacuoles represent?

ZLOTNIK: We spent several years doing all kinds of histochemical studies and all that we can say is that they contain empty space. I think the first contribution was Pattison's who drew attention to round masses in some vacuoles; and we found that, actually, there are two types of masses in these vacuoles: round bodies that might be mistaken for a kind of inclusion body; and homogenous pink matter filling the whole vacuole, which really looks to me like a localized necrosis of the neuron. One could thus postulate that there are two methods of producing the vacuoles in scrapie. One is due to blowing up of the cell, as if fluid were being pumped in, and the other is some form of localized necrosis of the neuron. We know that in every case of scrapie in a Suffolk sheep, when you open the skull, you find an increase in the cerebrospinal fluid. There is no morphological or chemical change in the fluid, but it is definitely increased in volume. One could say that this fantastic vacuolation seen in scrapie, in the ground substance and the neurons, has never been observed in any other disease, neither in animals nor in man. I agree that in many brains from human dementias, from Jacob-Creutzfeld and the Heidenhain syndrome, these localized areas of status spongiosus are found, but never in the same proportion as in scrapie; in some goats with the drowsy syndrome that go on for a very long time you might find that the thalamus is converted into one whole network of strands and empty spaces.

BROTHERSTON: What about B disease in monkeys; do you not get this vacuolation in the nervous system?

This may be relevant here because B disease is latent in monkeys.

ZLOTNIK: Of course, vacuolation exists in many diseases. In all brain tumors brain edema is a very common thing; status spongiosus is present in many conditions and vacuolation of neurons has been reported in many intoxications, but not this characteristic distribution and the same spread through the brain as is usually found in scrapie.

HADLOW: I would like to add to Dr. Zlotnik's comments in that the limited observations we have made certainly confirm the apparent alteration of the scrapie virus with passage in mice. We have observed similar changes in goats as well.

FIELD: This important point of the alteration in the character of the scrapie agent by passage through animals raises several very interesting possibilities, in particular, the possibility of interchange of genetic material with the host.

CASALS: A similar situation occurs when you have rabies virus in the street strain form and pass it intracerebrally in rabbits: you end up with a fixed form. Now, these two are absolutely identical immunologically. I do not think that in passing an agent through another species and altering the type of disease there is necessarily anything to be astonished at.

ZLOTNIK: What I am trying to draw attention to is that when you take the sheep strain and put it twice through mice, then put it once into a goat and finally back to the mouse, the species barriers do not exist because the incubation period in the mouse is unchanged. But when you take scrapie from a goat and put it twice through mice, then into a goat and then back to a mouse, the incubation period in the mouse is quite different, that is, not 5 months but 10 months. In the second case the strain originated from the goat and there was a reversal back to the goat pattern, but the strain of sheep origin did not revert on goat passage. I do not know about the drowsy goat type that Dr. Pattison has worked with, but, with the scratchy type, the evidence definitely suggests that it is not exactly the same scrapie as we deal with in Suffolk sheep.

BANG: Are the lesions characteristic enough and common enough so that one could do a pathogenesis study and follow the progress of the infection from one area of the brain to another?

ZLOTNIK: I compared lesions in the goat by subcutaneous and intracerebral routes and found that when it was given intracerebrally, which means it was forced to a certain degree, the first lesions that occurred were always in the mesencephalon, or

rather on the border between the pons and the mesencephalon in the tegmental region. When it was given subcutaneously the first changes were invariably somewhere in the medulla, usually at first in the inferior olive, and they were in the form of a transient status spongiosus. The possibility exists that the lesions that we see do not just appear and slowly develop; it might be that they develop and later decline when the body can cope with the fluid—let us assume that there is fluid in these vacuoles—and the brain adjusts itself until more of this vacuolation sets in; pressure of the vacuoles eventually produces necrosis and slowly leads to a state where the body cannot cope. When the balance between the defense mechanism of the cell and the drainage mechanism is upset, the clinical disease sets in.

HELMBOLDT: With this increased cerebrospinal fluid, is it known whether it is associated with increased pressure or is it just replacement?

ZLOTNIK: I wish I could answer this, but I do not think anybody has ever measured it.

BECK: Neuropathologists usually regard status spongiosus as a sign of edema and it occurs, as Dr. Zlot-

nik said, in quite a few conditions. Dr. Klatzo has shown most beautifully that in states of edema the astrocytes react within hours. It may be that the edema will subside, but the astrocytes remain; and that may be the explanation of this enormous astrocytic hypertrophy. Also, the choroid plexuses are very much enlarged, which is a further sign of increased production of cerebrospinal fluid.

FIELD: I think an interesting experiment would be to work with isogeneic animals which would accept things completely one from another, infect them with scrapie of another species, and see if the genetic material of the other species had been carried into them so that now they no longer accept from one another.

ZLOTNIK: What I have done is to give mice different strains. I have a type that I know has a long incubation period (based on previous data) of between 7 and 9 months and I have now reached the stage of mixing the mouse strain of Suffolk sheep origin with this sheep long period strain. I can thus distinguish the strains and I am looking forward to seeing what happens.

Experiments with Scrapie with Special Reference to the Nature of the Agent and the Pathology of the Disease

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INTRODUCTION

The transmissible agent of scrapie is generally referred to as a “virus,” although it may be well to bear in mind that, in spite of intensive study over the past few years, no virus unequivocally associated with the disease has yet been demonstrated culturally, visually or serologically. In these circumstances, it seems to me that the problem of identification of the scrapie agent should be attacked on the broadest possible front. By all means continue to apply orthodox virological techniques, but do not exclude other ideas and other methods merely because they are not obviously related to the assumption that scrapie is a virus-initiated disease.

Some of the unusual features of scrapie are well known, e.g., the long incubation period following experimental inoculation, and the considerable resistance of the transmissible agent to heat. Other unusual features are not so well known, and some are not easily disentangled from the literature. In this paper are presented some of my own observations that, as yet, have no obvious place in the scrapie jigsaw puzzle. These observations are not related to each other and are offered as a stimulus for speculation and discussion.

OBSERVATIONS

1. The Occurrence of Scrapie in Goats Following Intracerebral Inoculation With Normal Goat Brain.—These observations are summarized in table I. The 77 goats inoculated with 1.0 ml. of the supernatant fluid after light centrifugation of 10 percent suspensions in physiological saline of the 21 normal brains were control animals in various scrapie experiments

that were carried out over a 9-year period. It will be seen that five animals inoculated with four different brains developed scrapie, and that one animal inoculated with normal pancreas also developed the disease. By contrast, 155 goats inoculated with other control materials, e.g., saline, milk, urine, etc., and maintained in the same environment as control animals inoculated with normal brain, did not develop the disease.

TABLE I.—Occurrence of Scrapie in Goats Following I.C. Inoculation With Normal Goat Brain

Period covered by experiments	9 years
Number of normal goat brains tested	21
Number of goats inoculated	77
Periods of observation	22–36 months
Number of goats developing scrapie	5 from 4 brains
Incubation periods for the occurrence of the 5 cases of scrapie	17, 19, 23, 30, and 30 months

Scrapie also occurred in 1 of 3 goats 37 months after I.C. inoculation with normal goat pancreas.

Most of this information has been published (1, 2, 3).

2. Dialysis of Scrapie-Active Material.—By subjecting scrapie-producing material to dialysis it had been hoped to demonstrate that the agent was not dialysable, and that therefore it must be a particle of a size that could be determined by filtration experiments. However, it was found that scrapie-active material was present outside the dialysing membrane in four of six experiments. This work has been described in detail (4), and is summarized in table II.

TABLE II.—Occurrence of Scrapie in Goats Following Intracerebral Inoculation With Dialysed Scrapie Goat C.S.F. or Brain Material

Material dialysed	Number of experiments	Number of goats inoculated	Cases of scrapie
C.S.F.	3	14	5 (all same experiment)
10 percent brain supernatant plus radioactive marker	3	15	10 (some in each experiment)

NOTE.—In all experiments the material remaining inside the sacs produced scrapie.

3. Resistance of the Scrapie Agent to Formalin.—

In this work, 100 percent formalin=40 percent formaldehyde in water. Table III shows that scrapie goat brain homogenate was still capable of producing the disease after treatment with up to 20 percent formalin acting for 18 hours at 37° C. Table IV demonstrates the presence of active agent in small pieces of scrapie goat brain (originally taken for histological examination) after storage in 10 or 12 percent formalin at room temperature for up to 28 months.

The arrows in these tables indicate the occurrence of clinical scrapie in goats inoculated by the intracerebral route. The tables are reproduced by kind permission of the Editors of the Journal of Comparative Pathology and Therapeutics (5).

4. Shortening of the Scrapie Incubation Period in Goats Following Pretreatment With Normal Goat

TABLE III
Effect of Formalin on Scrapie Goat Brain

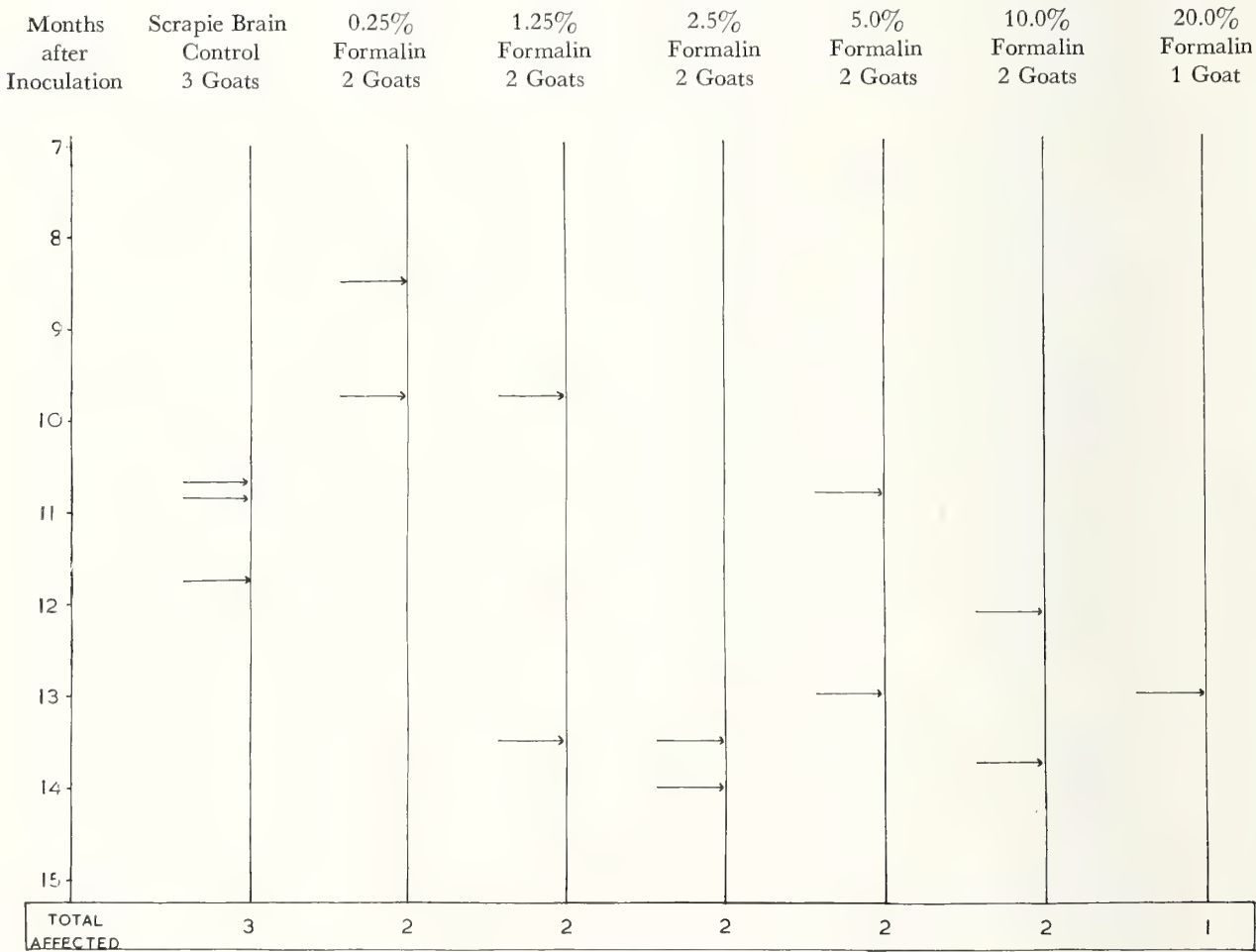
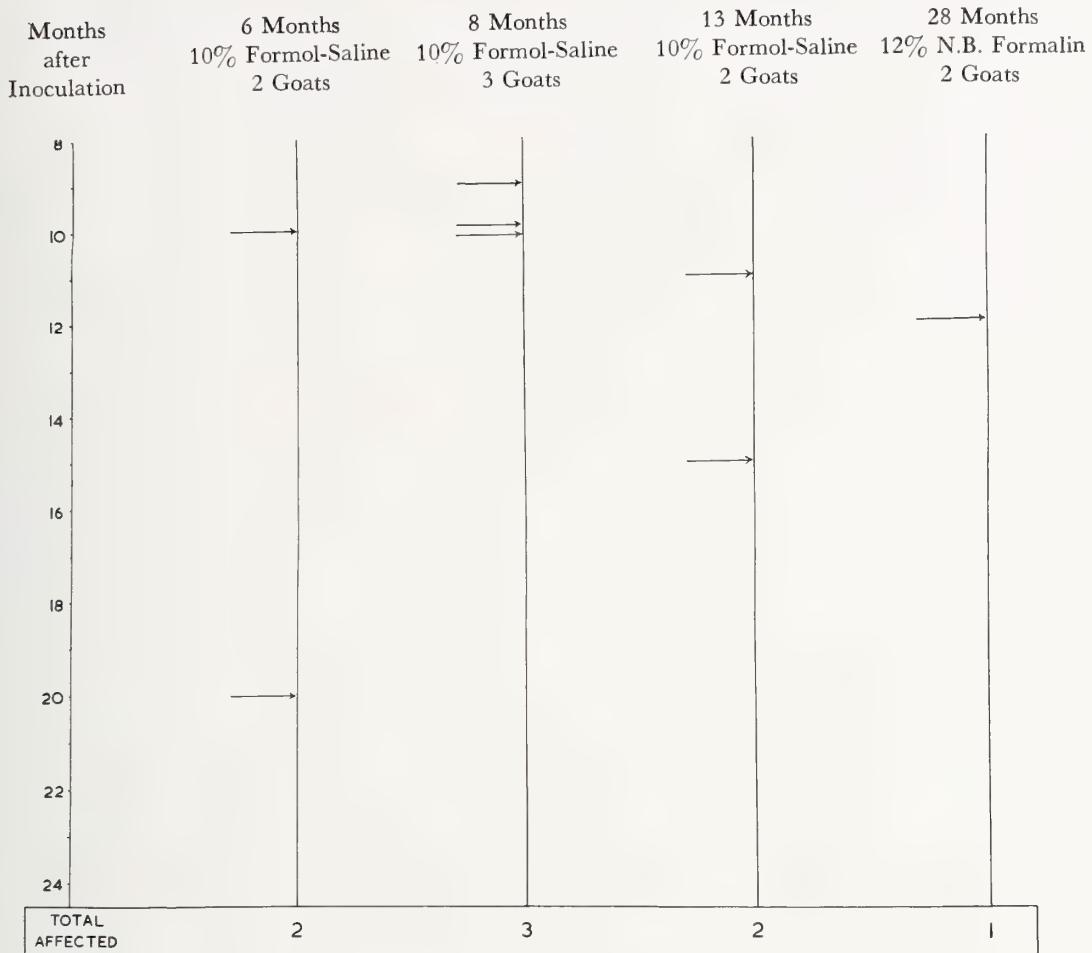


TABLE IV

I.C. Inoculation of Goats with Formalin-Treated Scrapie Brain



Brain.—In some respects there are similarities between the EAE agent and the scrapie agent, although, of course the histopathology of these two diseases is quite different. The possibility that some “immune” mechanism might be involved in scrapie was examined by “sensitising” goats with normal goat brain and then challenging them with scrapie. There was a significant shortening of the incubation period in a group of 15 goats (compared with 5 control animals) that had been given an intraperitoneal inoculation of 100 ml. of a dense suspension of normal goat brain 10 weeks before intracerebral inoculation with scrapie goat brain. This result is shown in table V. Details of this work have been published (6).

5. Passage of Scrapie Across the “Species Barrier.”—It has been noted that when scrapie is transmitted from one animal species to another (e.g., sheep/

goat, goat/mouse, mouse/rat, etc.), it is likely that there will be:

- A long incubation period.
- Atypical clinical signs in the recipient animal.
- Atypical histopathological lesions in the recipient animal.

Further, it has been noted that in the first passage between animals of the same species it is likely that:

- The incubation period will shorten and become fixed.
- The clinical signs and histopathological lesions characteristic for that species will be fixed.

These points are shown in table VI. The figures for incubation periods are averages from many different experiments. Experience indicates that, from experiment to experiment, a “fixed” incubation period may lengthen but will not significantly shorten. Apart from the modification reported under (4) above, all

TABLE V

I.C. Inoculation of Goats with Scrapie Goat Brain Ten Weeks After I.P. Inoculation with Normal Goat Brain

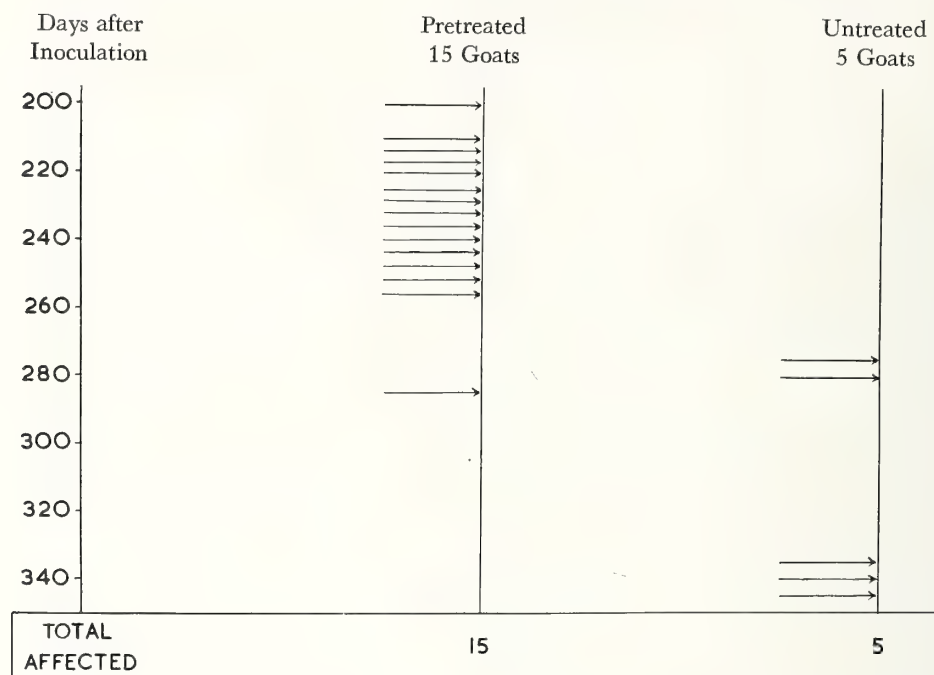


TABLE VI.—Intracerebral Passage of Scrapie Through Different Animal Species

Scrapie-affected donor	Recipient	Occurrence of scrapie in recipient (months)
SHEEP	SHEEP	5
SHEEP	GOAT ¹	18
GOAT	GOAT	8 Fixed
GOAT	SHEEP	9
SHEEP	MOUSE ¹	12
MOUSE	MOUSE	5 Fixed
MOUSE	SHEEP	24
GOAT	MOUSE ¹	7
MOUSE	MOUSE	4 Fixed
MOUSE	GOAT	10
MOUSE	RAT ¹	16
RAT	RAT	8 Fixed

¹ Atypical clinical signs and histology on passage to different species.

attempts to reduce a "fixed" incubation period in any animal species have failed.

6. The Occurrence of Local (Scratch) Lesions at the Site of Subcutaneous Inoculation of Scrapie Brain Suspension.—When the scratching type of scrapie agent (7) is inoculated intracerebrally into sheep or goats the scratch lesions seen when the clinical disease develops are likely to show a considerable degree of bilateral symmetry. The scratch stimuli that induce these lesions are generally believed to originate in the C.N.S. through degeneration of nerve cells. However, it has been noted that subcutaneous inoculation of a relatively large volume (5 ml. or more) of a dense suspension of scrapie brain may sometimes produce (several months after inoculation) an area of intense irritation at the site of inoculation. This has been seen twice in goats, in which species only a few subcutaneous inoculations with scrapie have been carried out, and in sheep. Table VII shows the occurrence of this type of lesion in 62 of 286 sheep that developed scrapie following subcutaneous inoculation of 5 ml. of a suspension of scrapie sheep brain behind the right shoulder. There was a marked tendency for the local lesion to occur in animals with short in-

cubation periods. The control goats referred to in table VII received multiple subcutaneous inoculations of normal goat brain.

TABLE VII.—Local Lesions at the Site of S.C. Inoculations of Scrapie Brain

Local lesions	Months after inoculation							
	6	7	8	9	10	11	12	13
Present	19	23	16	4	0	0	0	0
Absent	19	27	32	67	11	47	11	10

NOTE

Goats: Local lesions appeared in two goats 10 months after multiple inoculations (controls were negative).

Sheep: 286 cases of scrapie / 840 inoculated.

7. The Pathology of Experimental Scrapie in the Rat.—I would like now to describe briefly some work on the pathology of experimental scrapie in the rat, and to propound the heretical hypothesis that primary damage in scrapie is to the astrocyte, not to the nerve cell.

There now exists a considerable literature on the pathology of naturally-occurring and experimentally-produced scrapie. In different papers there are differences in emphasis, but there is general agreement that significant abnormalities are always found in the C.N.S. Most authors have mentioned one or more of the following (a) "Classical" degeneration of nerve cells (b) Vacuolation of nerve cells (c) "Extra-cellular" vacuolation (d) Astrogliosis. Several authors have drawn attention to the bilateral symmetry of abnormalities in paired structures. It is generally agreed that inflammatory changes and frank demyelination are absent. An impression that remains after examining the literature is that it is fairly generally believed that damage to nerve cells is primary and that astrogliosis and vacuolation (in its many forms) follow. To the best of my knowledge no author has attempted to explain the bilateral symmetry of the lesions.

In 1961 my colleague at Compton, Dr. R. L. Chandler, succeeded in producing scrapie in mice (8), and more recently, using a mouse-adapted strain of the agent, he has produced the disease in rats (9). I used rats for this study of the progressive pathology of the disease because:

(a) They are 100 percent susceptible to experimental scrapie.

(b) The disease progresses at the same rate in all animals given the same inoculum.

(c) Histological abnormalities, particularly of the astrocyte, are readily detected and become more marked and more extensive as the preclinical and clinical stages of the disease advance.

In sheep and goats, by contrast, the disease progresses irregularly in any one group of inoculated animals, so that it is virtually impossible to make a step-by-step study of the pathology. Also, the astrocyte and its processes stain so well in normal sheep and goats that early hypertrophic changes in the diseased animal are extremely difficult to detect.

Two groups of rats were inoculated intracerebrally with either normal rat brain or scrapie rat brain, and pairs of animals from each group were killed for examination at 14-day intervals from the time of inoculation until the last stages of the clinical disease (224 days after inoculation). After fixation in 10-percent formol-saline, the brain of one of each pair was cut sagittally about 1 mm. to the left of the midline, and the brain of the other was cut transversely at the level of the hypophysis. Sections from each brain were stained with haematoxylin and eosin and by Cajal's gold sublimate method.

The histological abnormalities noted were:

(a) *Hypertrophy of astroglia*, first visible 84 days after inoculation (78 days before the earliest clinical abnormality) and limited at that time to the thalamus close to the ventricle floor and to the pyramidal area of the hippocampus. This hypertrophy of astroglia became progressively more widespread, and in the latest stages of the disease virtually the whole brain, including the cerebellum and the cerebral cortex, was involved.

This astroglial hypertrophy is illustrated in figure 1. Section A is from a rat inoculated with normal brain, and B, C, and D are from rats inoculated with scrapie brain showing advancing stages of the disease. All sections are from exactly the same area of the hippocampus.

(b) "*Extra-cellular*" vacuolation, that followed the hypertrophy of astroglia and occurred only in those areas where there was astroglial hypertrophy. Thus, hypertrophy of astroglia was first seen in the thalamic nuclei 84 days after inoculation, but vacuolation was not detected until 2 weeks later and was confined to this area. As the disease progressed the areas showing vacuolation expanded, but their strict identity with areas of astroglial hypertrophy was unmistakable.

(c) *Degeneration of nerve cells*. The changes noted were pyknosis, shrinkage and, rarely, vacuolation. These also followed in time the hypertrophy of

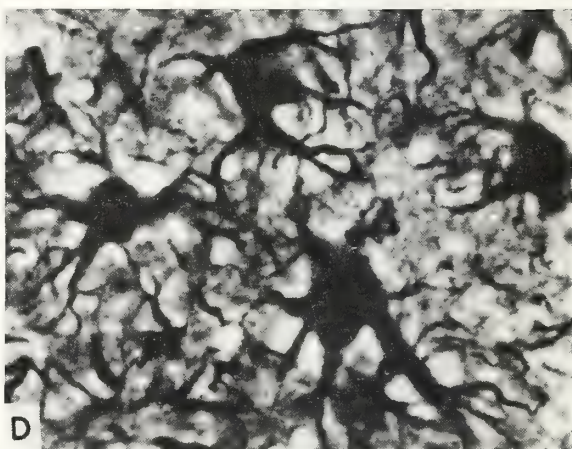
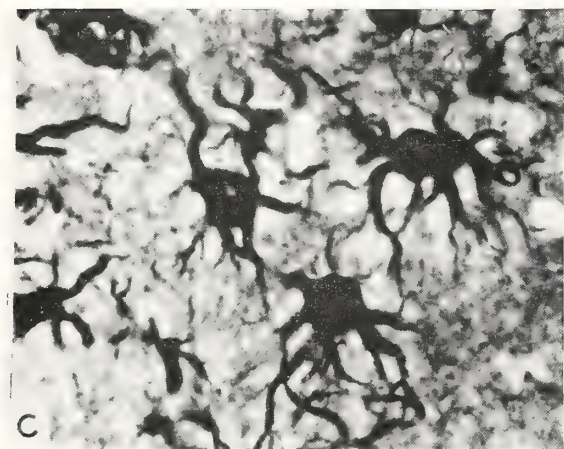
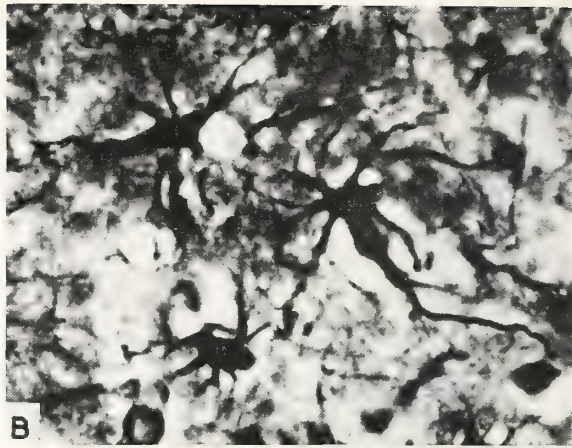


FIGURE 1.—Sections from the same area of the hippocampus of four inoculated rats. A is from a rat inoculated with normal brain. B, C, and D are from rats inoculated with scrapie brain, taken at increasing times after inoculation, showing progressive hypertrophy of astroglia. The magnification is the same in each case. Cajal \times about 800.

astroglia and, as in the case of “extra-cellular” vacuolation, they were clearly associated with it.

(d) *Bilateral symmetry of the lesions.* The precise bilateral symmetry of the lesions (astroglial hypertrophy, “extra-cellular” vacuolation, and nerve cell degeneration) in paired structures throughout the brain was an outstanding feature at all stages of the disease.

(e) *Dilation of the ventricles.* Some degree of dilation of the ventricles is fairly commonly found in rat brains, but in this series of animals it was more marked in scrapie-affected than in normal animals, especially late in the disease.

On the basis of these studies in rats, and taking into account earlier studies on the pathology of scrapie in sheep, goats, and mice, I suggest the following explanation for the pathological changes in the C.N.S. in scrapie: That primary damage is to the astrocyte, and

that nerve cell degeneration and vacuolation in its many forms are secondary changes consequent upon abnormal metabolism of the astrocyte.

This hypothesis is based on:

(a) The time sequence for the occurrence of abnormalities in rats, i.e., astrocytic hypertrophy preceding degeneration of nerve cells and “extracellular” vacuolation.

(b) The strict spatial relationship between astroglial hypertrophy, nerve cell degeneration and “extra-cellular” vacuolation.

(c) The tendency for ventricular dilation, indicating fluid imbalance within the brain.

(d) Recent concepts (10), based on electron microscope studies, of astroglia and of their intimate association with blood vessels, cerebrospinal fluid, and nerve cells. Thus, it is now believed that astroglia have

functions that are similar in some degree to the extra-cellular space of other tissues, that they are in osmotic equilibrium with cerebrospinal fluid, that they represent a pool of water and electrolytes between the blood plasma and the neurons, and that they may be the site of the blood-brain barrier.

Speaking personally, the strict bilateral symmetry of the C.N.S. lesions in scrapie has always been an insuperable obstacle to my acceptance of the conception of scrapie as being caused by an "orthodox" virus with a primary pathogenic action on nerve cells. I cannot visualize a virus with so precise a primary action. If, however, one postulates that the primary dysfunction is of astroglia—perhaps initiated at the level of the blood-brain barrier—then bilateral symmetry of lesions, reflecting the bilateral symmetry of the blood supply, is readily understood.

SUMMARY

Comment is made on a number of unrelated experimental observations that emphasize the unusual nature of scrapie as a disease and of the transmissible agent associated with it, namely:

1. The occurrence of scrapie in goats following intracerebral inoculation with normal goat brain.
2. Dialysis of scrapie-active material.
3. The resistance of the agent to formalin.
4. Modification of the incubation period following pretreatment with normal brain.
5. Transmission of the disease across the "species barrier."
6. The occurrence of local (scratch) lesions at the site of subcutaneous inoculation of scrapie brain.

A brief description is also given of the progressive pathology of experimental scrapie in the rat, and the postulation is made that primary damage in scrapie may be to the astrocyte and that nerve cell degeneration and intra- and extra-cellular vacuolation may be secondary changes consequent upon abnormal metabolism of the astrocyte.

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DISCUSSION

GAJDUSEK: What type of dialysis membrane did you use in your experiment?

PATTISON: Visking tubing, A.P.D. 2.4 mμ.

GAJDUSEK: As a control you worked with a radioactive labeled protein?

PATTISON: It was radioiodinated serum albumen.

GAJDUSEK: And this did not come through?

PATTISON: Well, the thing with this particular marker is that you do get leakage of what is believed to be free iodine, but this is a constant amount and is very easily measured, and control bags were always put up. We fortunately made one or two faulty bags and we went through this marker system before we ever set up the experiment. If you have a slight leak in the bag, the difference in radioactivity is extraordinary.

GAJDUSEK: This is obviously crucial. We cannot simply sit down and settle for the fact that the scrapie agent may be or may not be passed through a dialysis membrane. If it is, this is not a viral agent that we are transmitting. We may only be activating a latent virus.

KIES: This business of dialysis is terribly complex. You cannot say that something is dialyzable or not dialyzable. I will agree with you, though, that if it goes through any kind of Visking tubing it is not what people normally think viruses are. But, in general, you cannot say that a given molecular

weight material is dialyzable and another is not; it is a relative thing. We got into the same argument with regard to the encephalitogenic protein which, of course, is much smaller than a virus.

PATTISON: I think we have to be very cautious in our conclusions, for the most extraordinary results have been reported, and the conditions of dialysis, the type of material that you use, etc., are most important. I do not think that you can make an absolute distinction between a large and a small, but the point that we reached is that we suggest that this agent is probably unusually small.

GAJDUSEK: I am still not satisfied. We certainly know the difficulty of making perfect membranes and, in fact, perfect membranes cannot be made. We also know that there is a range of particles that get through and this range can be calculated. On the other hand, are we now saying that sterilizing membranes of a pore diameter much below that of the size of an agent are inadequate to insure biological sterility? In other words, if this uncertainty applies to scrapie, then it should also apply to poliomyelitis virus and other viral agents as well, yet membranes have long been used for sterilizing biological material.

KENYON: There are a number of ways of getting at the size other than using dialysis. In addition, one could fractionate using either charged or noncharged supporting media and then see which fraction contains the agent.

LEADER: I was thinking back to work done with tobacco mosaic virus about 8 or 9 years ago in which the virus was broken up with detergents and little pieces of protein taken out, and when put back together in the proper concentration these reassociated into infective 300 millimicron particles all by themselves. In brain tissue suspensions you have bipolar molecules and lipid soluble and water soluble material all mixed together and I wonder if it is possible that there is some kind of detergent-like action going on which could disassociate and reassociate these particles under natural circumstances.

POSKANZER: Several other points come to mind from Mr. Pattison's very provocative paper. First of all, in the central nervous system we see this astrocytic change in one condition particularly, hepato-toxic encephalopathy, which we think is due to metabolic derangement, presumably from the presence of ammonia or some other metabolite in the circulating blood as a result of failure of the liver

to remove it. This disease can go on to a chronic form as well as its usual acute one and may go on for many months. If the scrapie agent is a dialyzable substance and does not produce antibody and is extremely difficult to destroy, then it might very well be an inorganic substance. It is one kind of substance that would fit all these criteria. Suppose this substance acts by poisoning an enzyme system. This would be consistent with the symmetrical pattern of lesions in the brain, which is consistent with a metabolic process rather than an infective one. It could be similar, for example, to the situation which arises in carbon monoxide poisoning, where there is an initial poisoning, a period of recovery, and then, 3 or 4 weeks later, death, presumably as the end-result of the poisoning of the production of some enzyme. If you want to stretch this analogy a little bit further, it could be assumed that there is a hereditary predisposition to damage by this inorganic molecule, as there is with certain other chemicals, so that not every sheep gets it.

GAJDUSEK: One point I would like to emphasize, and that is Mr. Pattison's suggestion that astrocytes may be primarily involved in scrapie. I think this is an extremely important suggestion. Also, in the disease we know so little about yet, kuru, the astrocytosis is so immense that it has been regularly suspected of being the primary phenomenon in the disease.

FIELD: May I say that it is a long-standing hypothesis in multiple sclerosis that the primary site of the lesion is in the astrocytes. This was put forward with considerable vehemence by von Mueller in 1904, and all observers of multiple sclerotic lesions are agreed that the astrocytic response is enormous. It is far more than looks necessary for the amount of degeneration that has gone on. One can imagine that some viral agent acts primarily by stimulating the astrocytes; and we know that neuroglia are concerned with the maintenance of the integrity of the myelin sheaths, so that one can imagine, if this process is more accentuated in certain foci for one reason or another, that focal demyelinating plaques may occur.

MARGOLIS: I am still impressed by the neuronal lesions, and not so impressed by the changes in the astrocytes. We get so many examples of tremendous glial hyperplasia without seeing this striking neuronal vacuolation.

GAJDUSEK: The question here, I think, is whether the astrocytic hyperplasia may not be causing the neuron changes.

PATTISON: This was the basic conception, that the first detectable thing morphologically was the astrocytic hypertrophy, followed by the extracellular

vacuolation and the neuron changes which were spatially strictly related to it. This relationship is quite easy to follow in the mouse because it occurs in very special areas, the hippocampus, the thalamus, and so on; you can follow the changes as they progress, always bilaterally symmetrical.

Progress Toward the Isolation and Characterization of the Scrapie Agent

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INTRODUCTION

The successful transmission of scrapie to mice (1-5) gave a new lease of life to research on scrapie. It became much easier to carry out quantitative work by using large numbers of mice in titration experiments (2, 3, 6, 7). In the Biochemistry Department at Compton, where an attempt is being made to effect an isolation of the scrapie agent, it soon became apparent that the bulk of the infective agent was to be found in the particulate fractions of the cell (6) and was stable under normal conditions of cellular fractionation (8). Further application of techniques of cellular fractionation showed that the major portion of the infective agent was located in the mitochondrial-lysosomal fraction of the cell (9). It was also found (10) that the development of the disease was associated with the appearance of elevated levels of lysosomal enzyme activities in brain.

In the present communication, it is proposed to summarize as briefly as possible further progress that has been made recently in the course of attempts to concentrate and characterise the agent. Most of the detailed procedures used in studying the heat stability and chromatographic behavior of the agent have been published recently (11), and for this reason it is proposed to omit an experimental section from the present paper. All experiments have, however, been carried out using the Chandler mouse strain, and all titrations are based on the weight of brain from which the relevant material was originally derived, and on inocula of 0.02 ml. volume.

RESULTS

Saline extracts of scrapie mouse brain were heated at various temperatures between 50° and 141° for

both 10 and 60 minutes. Apart from a drop in titre in material heated at 62.5°, it can be seen that rapid loss of infectivity does not occur until the scrapie extracts are heated above 87.5° (table I).

TABLE I.—Action of Heat on Saline Extracts of the Scrapie Agent

Temperature used (°C.)	Residual titre (ID ₅₀) after heating for—	
	10 minutes	60 minutes
20°	5.5	5.5
50°	5.3	4.8
62.5°	4.3	4.4
75°	4.9	5.1
87.5°	4.5	4.0
100°	2.1	<1.7
118°	<1.6	—
141°	—	—

Table II summarises the results of experiments where the scrapie agent was fractionated by chromatography on DEAE-cellulose, both before and after dispersal of extracts by ultrasonic vibrations. After the ultrasonic treatment, a full titre passes rapidly through the DEAE-cellulose columns. Very similar results were obtained when scrapie extracts were chromatographed on calcium phosphate.

The results of preliminary attempts to release the agent from proteinaceous debris are summarised in table III. All these attempts merely result in the conversion of the agent into a form even more readily sedimentable than previously. However, treatment with Arcton-113 (table IV) at 0° to 5° does seem to remove a large proportion of the debris without loss of overall titre. However, as shown in table V, the agent may

TABLE II.—Chromatography of Intact and Ultrasonicated Scrapie Mitochondrial Preparations on DEAE-Cellulose

Intact mitochondria. Material eluted with—	ID ₅₀	Ultrasonicated mitochondria.	
		Material eluted with—	ID ₅₀
0.01 M phosphate buffer	3.2	0.01 M phosphate buffer	5.0
0.1 M NaCl	—	0.1 M NaCl	2.5
0.15 M NaCl	—	0.15 M NaCl	<1.7
0.3 M NaCl	<1.7	0.3 M NaCl	2.5
1.0 M NaCl	3.0	1.0 M NaCl	2.2
Titre of material applied to column		Titre of material applied to column	
4.5		4.8	

TABLE III.—Sedimentation Behaviour of the Scrapie Agent After Treatment with Ultrasonic Vibrations, Heat or Neutral Detergent

Treatment	Initial titre (ID ₅₀)	Subsequent titre in material sedimenting at:			Titre in final supernatant
		500 g.	10,000 g.	100,000 g.	
Ultrasonics	5.3	—	5.2	1.7	—
Heat (75°)	5.2	5.0	2.0	—	—
Tween-80	4.5	4.5	3.2	—	—

TABLE IV.—Treatment of a Scrapie Brain Homogenate with Arcton-113 at 0° to 5°

Material	Titre (ID ₅₀)	Protein nitrogen content (mg.)
Initial homogenate	5.7	3,987
High-speed pellet from a single Arcton treatment	6.2	220
High-speed pellet from a second Arcton treatment	5.5	44

TABLE V.—Attempted Chromatography of an Ultrasonically Dispersed Pellet Derived from a Scrapie Brain Homogenate Extracted Twice with Arcton-113

Chromatography on DEAE-cellulose		Chromatography on CM-cellulose	
Material eluted at pH 7.5 with—	Titre (ID ₅₀)	Material eluted at pH 5.0 with—	Titre (ID ₅₀)
0.01 M phosphate buffer.	4.0	0.01 M citrate buffer.	0.8
0.15 M NaCl		0.1 M NaCl	0.7
1.0 M NaCl	2.8	0.2 M NaCl	
		0.4 M NaCl	
		0.8 M NaCl	0.8
Initial titre of material applied.	5.5	Initial titre of material applied.	3.7

DISCUSSION

The experiments on the heat stability of the scrapie agent suggest: (1) that the bulk of the agent exists in a single heat-stable form; (2) that a double-stranded nucleic acid, probably DNA, is a component of the agent. The slight irregularity in the heat-sensitivity curve observed with material heated to 62.5° probably arises from the action of enzymes, some of which would be relatively slowly inactivated at that temperature. Similarly, incubation of scrapie suspensions at 37° in tris buffer (pH 7.5) results in a progressive loss of titre (12).

The results of the chromatographic experiments are similar in many respects to those obtained Mould et al. (7). They do at least show that the scrapie agent is not found abundantly in association with mitochondria by virtue of a comparable size and density. In fact, the agent remains intact when the mitochondria are shorn into small pieces by ultrasonic vibrations and can then pass quantitatively through columns of DEAE-cellulose. The results of experiments using heat or a neutral detergent (table III) show that the agent does in fact bind very tenaciously to denatured lipoprotein debris, and these early attempts failed completely to release it. However, treatment with Arcton-113 (table IV) at 0° to 5° has given much more promising results, and the bulk of the debris seems to be removed by this procedure. The agent still seems to exist as a complex with low-density material, however, and attempts are at present being made to re-

still exist largely as a complex with lipoprotein debris and cannot be fractionated satisfactorily on columns of DEAE- or CM-cellulose. It also behaves as a complex with lipoprotein debris when equilibrated in a sucrose density gradient (12).

move the residual debris by various enzymic procedures and treatment with detergents.

As regards the nature of the scrapie agent, the question is still open. It has never been possible to obtain the bulk of the infective material in other than a particulate form, even though the agent is still predominantly associated with material of a density lower than other known viruses. The hypothesis that best fits the present facts seems to be that the bulk of the scrapie agent is a small DNA virus in the 20 to 40 $m\mu$ size range, binding avidly to tissue debris perhaps because it has a basic surface (contrast the binding properties of the agent to DEAE- and CM-cellulose, table V). The evidence leading to this hypothesis may be summarised as follows:

(1) The heat stability of the scrapie agent resembles the heat denaturation curves of many native DNA's, and would be difficult to reconcile with the presence of a single-stranded RNA.

(2) Native DNA reacts very slowly, if at all, with formaldehyde. The resistance of the scrapie agent to formalin (13) would again be difficult to reconcile with the presence of a single-stranded RNA species. Of course, the rare double-stranded form of RNA (14) would be much more similar to DNA in its stability to physical and chemical agents.

(3) The rate of synthesis of DNA is elevated in the brains of clinically affected scrapie mice (15).

(4) In a recent experiment on the filtration of the scrapie agent (16) some infective material passed through a 50 $m\mu$ filter but not through a 25 $m\mu$ filter.

(5) Particles in the 20 to 40 $m\mu$ size range are sometimes found in excess in electron micrographs of scrapie material. However, these particles may not be directly associated with the scrapie agent and are possibly lysosomal debris.

(6) The firm binding of the agent to particulate debris itself of a small size after, for instance, ultrasonic treatment, suggests that the agent cannot be a large virus.

(7) It behaves like many other viruses when treated with Arcton-113.

On the other hand, although there does not seem to be any positive evidence against this hypothesis, some experimental work suggests that a small portion of the infective agent may exist in an anomalous form. If the information for the reproduction of the scrapie agent has become incorporated into the genome of the host cell as appears to be the case in the polyoma-transformed cell (17), then the effective size of the agent could become much smaller than 20 $m\mu$.

SUMMARY

Early experiments on mouse scrapie showed that the agent binds strongly to proteinaceous tissue components, particularly those found in the mitochondrial fraction of the cell. For this reason, cellular fractionation and simple chromatographic procedures do not by themselves permit the agent to be concentrated to any worthwhile extent. However, treatment with Arcton-113 at 0° effects a partial release of scrapie infectivity from the debris, with an enrichment factor of about 100. It is possible that the remainder of the debris can be removed by the action of detergents and/or enzymes.

The precise nature of the scrapie agent still eludes investigators. It may be smaller than any known virus, but most of the scanty evidence at present available would suggest that it is more probably in the 20 to 40 $m\mu$ size range with a double-stranded nucleic acid component, probably DNA.

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The Scrapie Eradication Program

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Scrapie is an insidious disease of sheep and goats caused by a transmissible agent, characterized by a long incubation period and a progressive degeneration of the central nervous system which causes the affected animals to rub and scratch, become debilitated and incoordinated and terminates in death in nearly all, if not all, cases.

The disease has been known in Great Britain and countries of Western Europe for more than 200 years.

Scrapie Control and Eradication Programs in Various Countries

Countries in Which Scrapie Has Been Reported

Although the history of scrapie, its origin and epidemiology are distorted by hearsay and conjecture, early workers expressed considerable interest in the disease and it has been reported in Australia, Austria, Canada, England, France, Germany, Hungary, Iceland (where a similar disease is called *rida*), India, New Zealand, Norway, Poland (Prussia), Scotland, Spain, Wales, and the United States.

Eradication Efforts in Australia, Canada, New Zealand, and the United States

In Australia and New Zealand scrapie was first reported in 1952 and is believed to have been newly introduced by imported British sheep. Eradication programs including quarantine and slaughter procedures were immediately instituted. In New Zealand, a second outbreak, apparently an extension of the previous one, occurred in 1954. Officials in both countries consider their efforts to eradicate scrapie have been successful, and that the disease no longer exists in either country.

Scrapie was first reported in North America in 1939 in Canada. The first case reported in the United

States occurred in Michigan in 1947. Both outbreaks are believed to have been associated with the importation of British sheep. Regulatory officials in the United States have worked closely with those in Canada as scrapie problems in the two countries are similar and are closely associated. The two countries have very similar Scrapie Eradication Programs.

Control of Scrapie in Other Countries

In certain countries where scrapie is endemic, owners attempt to reduce the incidence of the disease and the stigma of having an infected flock by slaughtering selected animals of certain bloodlines. These individual efforts do not represent organized eradication programs. Such measures are often helpful in suppressing the disease but seldom eradicate scrapie.

Losses Due to Scrapie

Losses in the United States (fig. 1)

Since 1947, scrapie has been diagnosed in 138 flocks in 26 States—as shown on the map. Round number estimates through June 30, 1964, indicate 80,000 sheep have been slaughtered with \$2,100,000 Federal indemnity and \$1,200,000 State indemnity, or a total of approximately \$3,300,000 in indemnity payments to flockowners. It is impossible to determine the losses which may have occurred in the United States from scrapie had the Cooperative Scrapie Eradication Program not been initiated, because many of the animals destroyed under the program would have developed and disseminated scrapie. Therefore, it is reasonable to believe the program has prevented the disease from becoming more widespread in the Suffolk and Cheviot breeds and prevented spread to other breeds in the United States.

SCRAPIE REPORTED IN THE UNITED STATES

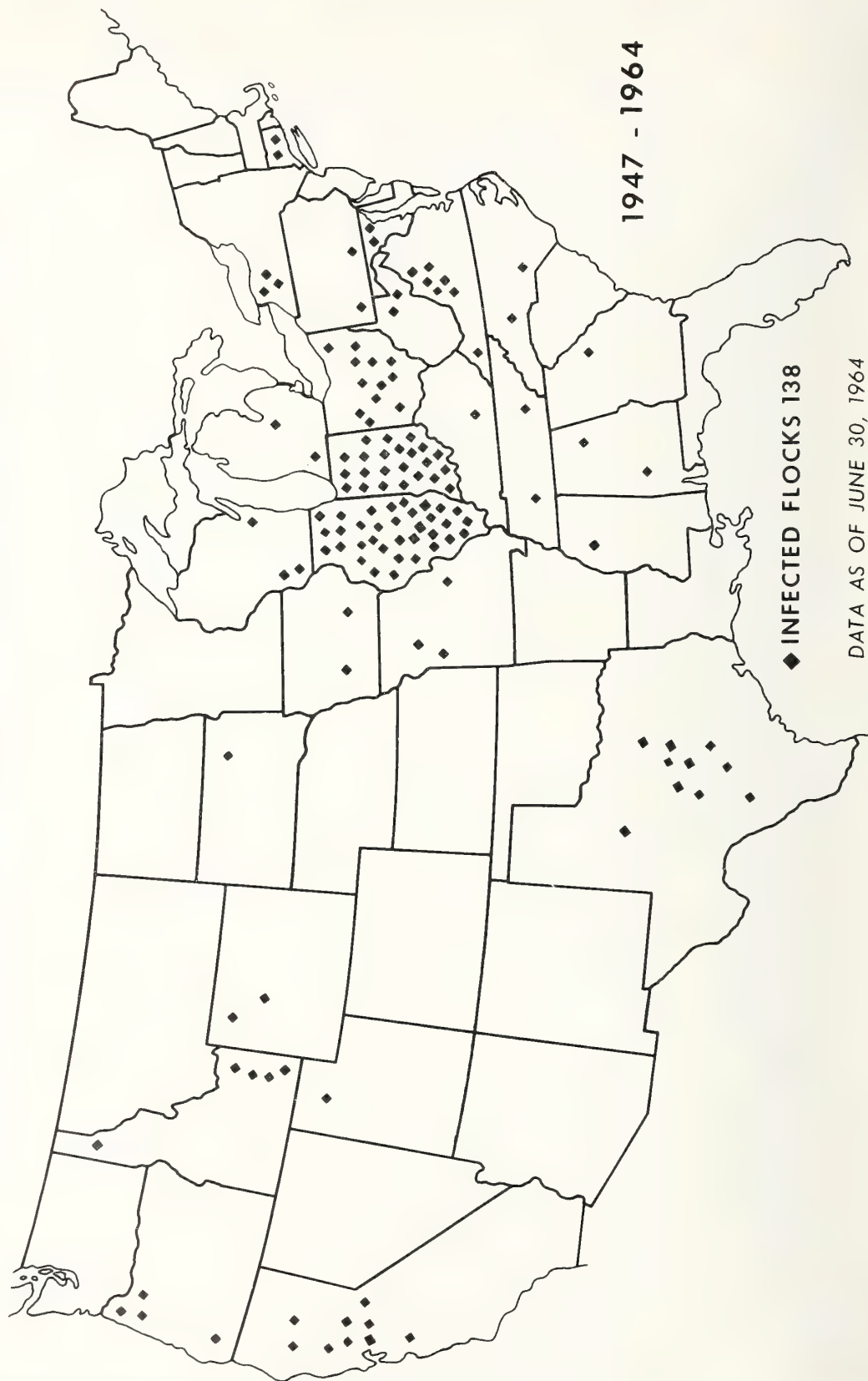


Table I gives a breakdown, by fiscal year, of the 138 infected flocks and 229 affected sheep. There were 6 Cheviot flocks—3 in Ohio, 2 in Illinois, and 1 in Oregon—and a total of 12 Cheviot sheep found to have the disease. The remainder of the known affected sheep, 217, were Suffolks. The affected sheep—63 rams and 166 ewes—were found in 106 counties in 26 States.

Losses in Other Countries

France—Estimates by different workers are 4, 4, 7, 15 to 25, and 10 to 30 percent of infected flocks.

Iceland—3 to 16 percent per year in infected flocks.

India—The incidence in 33 villages was given as 1 to 10 percent.

Britain—One British worker indicated that many flocks may be considered as free, that is, no manifest cases for at least 5 years; in many the incidence is sporadic (1 percent) or low (1 to 3 percent); in some it is medium (4 to 10 percent); and in a few

flocks it is high (10 percent), and may exceed 20 percent. In severely infected flocks 25 to 40 percent of the second crop ewes may die from the disease. Another reported that in 19 infected flocks studied, ewes had about a 10-percent chance of developing scrapie during their lifetime (an average of about 4½ years).

Indirect Losses from Scrapie

We are also given to understand that the heavy culling of scrapie families, disorganization of breeding programs, difficulty of becoming free again in a heavily infected flock, loss of reputé, and resistance of buyers to purchase breeding sheep from infected flocks, all combine to make scrapie a dreaded disease. While some breeders manage to live with scrapie and, with varying success, to cull against it, others abandon the struggle and dispose of the flock (thus further disseminating the disease), often at a heavy financial loss.

TABLE I

SCRAPIE REPORTED 1947-1964

FISCAL YEAR	AFFECTED FLOCKS			AFFECTED SHEEP			SEX		STATES	COUNTIES
	CHEVIOT	SUFFOLK	TOTAL	CHEVIOT	SUFFOLK	TOTAL	RAMS	EWES		
1947	0	1	1	0	9	9	0	9	1	1
1953	0	10	10	0	45	45	2	43	3	9
1954	0	3	3	0	6	6	1	5	2	3
1955	1	10	11	3	11	14	5	9	8	11
1956	1	22	23	2	37	39	14	25	9	20
1957	0	12	12	0	13	13	7	6	6	12
1958	1	6	7	4	6	10	5	5	6	7
1959	0	11	11	0	12	12	3	9	8	11
1960	1	12	13	1	13	14	7	7	9	22
1961	1	8	9	1	10	11	2	9	6	9
1962	1	12	13	1	27	28	8	20	4	13
1963	0	11	11	0	11	11	5	6	6	11
1964	0	14	14	0	17	17	4	13	3	11
1965										
1966										
1967										
1968										
TOTAL	6	132	138	12	217	229	63	166	26	106

DATA AS OF JUNE 30, 1964

Percentage of Affected Sheep Within Age Groups at Time of Death or Destruction

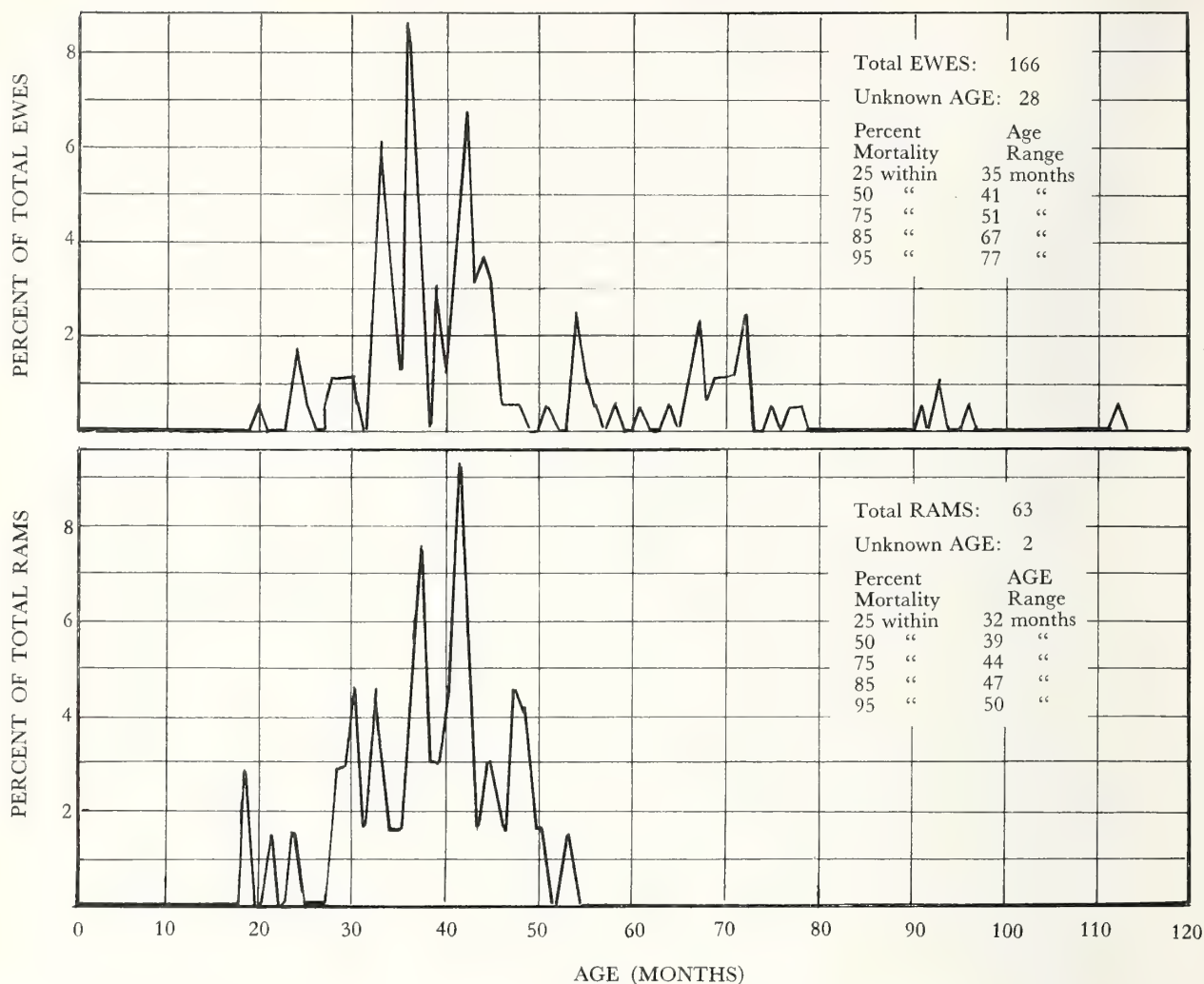


FIGURE 2

Figure 2 reflects the age, in months, of scrapie-affected rams and ewes at the time of death or destruction. On this graph the numbers of affected ewes and rams are expressed in percentages of the total of each which gives a rather interesting comparison between rams and ewes. The curves up to about 50 months of age are quite similar. However, beyond that point the ewes carry on rather strongly to 80 months and even on up to 112 months or more than 9 years of age. The rams, on the other hand, drop off abruptly at near 50 months of age. One is inclined to suspect that this is because rams are culled, die of other causes, or are otherwise removed from risk earlier than ewes and thus have less opportunity to exhibit the disease at older ages.

Development of the Cooperative State-Federal Scrapie Eradication Program

The objectives of the Scrapie Eradication Program are prevention, control and eradication of the disease.

Only two basic disease eradication principles are applicable in attaining these goals. These are preventing exposure of susceptible animals and/or increasing their resistance.

Research efforts to produce a vaccine to increase resistance have been discouraging. If either breed or individual animal resistance occurs, it might be possible to develop strains of scrapie-resistant sheep. Even if possible, this would be a very long and laborious

undertaking and not necessarily compatible with breeding programs designed for other purposes.

Preventing exposure of susceptible sheep is the only presently known method of avoiding additional outbreaks. Research workers maintain that the disease may spread from parent to offspring or by direct and indirect contact. Until research quantitates the relative danger from each, program direction must consider appropriately modified conventional methods which embrace both possibilities of spread. Since the scrapie agent has been demonstrated to be present in many tissues in clinically affected animals, it is clear that the visibly affected sheep must be destroyed. There is no diagnostic test for the disease in the live animal not showing signs; consequently, there is no way of knowing how many exposed animals may harbor the infectious agent. This factor, linked with the long incubation period, has necessitated the slaughter of exposed animals. The consensus has been that the immediate progeny of exposed animals may also be dangerous.

The program adopted in 1952 in California included laboratory confirmation of the disease in animals showing clinical signs of scrapie, quarantine and slaughter of all sheep and goats in the infected flock, and tracing and slaughter of all exposed animals moved from the flock and their immediate progeny. The 55 premises involved were cleaned and disinfected. These procedures were quite effective and now more than 12 years have passed with no extension of infection from these outbreaks. California has since suffered seven additional outbreaks—not associated with those in 1952.

Indemnities for slaughtered animals have been paid in accordance with State and Federal regulations. Federal indemnity was increased in 1954. It does not exceed 50 percent of the difference between appraised and net salvage value of each animal and is limited to \$25 a head for grade and \$75 a head for purebred animals. The amount of State indemnity paid is governed by State policy and regulation.

The consensus is that the program is holding the disease well in check: however, it is difficult to predict that the present program will achieve total eradication based on the present knowledge of the disease. One can point to evidence of the program's success in California and Indiana, where the results have been quite encouraging.

Scientists, in sharing their considerable knowledge and experience with us, have been very helpful from a control and eradication viewpoint. From this point of view I should like, if I may, to very briefly com-

ment on some of the significant aspects of this information. First, there has been some question as to whether a transmissible agent is involved in scrapie. The proof as to whether such an agent causes scrapie has been clearly demonstrated by taking material from the affected animal, introducing it into a second animal, and reproducing the disease in the latter. The disease can be passed through an apparently unlimited number of serial passages. Secondly, research workers at several institutions have taken material from scrapie-affected sheep and produced the disease in animals of a different species—goats, mice, rats, and hamsters. This further challenges the theory that the cause of scrapie is merely a simple autosomal recessive gene. Research findings presented at this symposium, and previously, demonstrate that contact transmission does occur in scrapie and that, although the transmissible agent is more resistant to heat and other abuses than many viruses, it performs generally as a filterable virus and should be considered as such. Research work presented further suggests that some manner of transmission takes place from parent to offspring and that the frequency of such occurrence is probably greatly influenced by genetic natural susceptibility or resistance.

Studies of Outbreaks in the United States

Each outbreak is carefully studied in an attempt to establish the epidemiology of the disease. Particular attention is given to signs, laboratory findings, age, sex, breed, history, and pedigree of each affected animal, movements from infected flocks, and the possible source of the outbreak.

During the nearly 12 years the eradication program has been in effect in this country it has not been possible to identify the source of some outbreaks. However, the majority fall into a pattern and indicate spread from a limited number of common foci. Once these foci are identified and eliminated they cease to disseminate the disease further.

We, of course, attempt to compare and correlate our field observations with information developed by research.

It is an accepted fact that the scrapie virus is widely disseminated in the affected animal and that scrapie can be produced in sheep, goats, and laboratory animals inoculated by various routes with different tissues from affected animals, or by feeding animals tissues taken from affected sheep, goats, or mice.

The matter of immediate concern is—how the disease spreads naturally from one sheep to another.

Obviously, it does, or scrapie would have eliminated itself centuries ago—or, perhaps, would have never existed. The possibilities to be considered are that the disease spreads from one animal to another by contact, either directly through close association or through breeding, or indirectly through the medium of infected premises; or perhaps from the ram or the ewe or both to their progeny. If the ram transmits the disease to his progeny, is the transmissible agent present in the semen? Or it is attached to genes or chromosomes, using them as a means of transportation? In the case of the ewe, is the virus within the ovum? Is in utero transmission a factor? Does the lamb acquire the virus from the ewe's milk? Or does the intimate contact of ewe with lamb during early life provide the exposure? What are the genetic factors involved? These questions, by regulatory veterinarians, must in the end be answered by research workers; and such answers are not easily obtained.

Importance of Transmission Along Bloodlines

Both experimental and field evidence suggest that scrapie can spread from the dam, and perhaps the sire, to the progeny. Many breeders who have had experience with the disease believe this to be true. Research workers reported during the Scrapie Seminar held in Washington in January 1964, that when two affected sheep are mated their progeny nearly always develop the disease (1). A much higher proportion of the progeny of affected ewes develop scrapie than those of affected rams. Stamp and Dickinson and their co-workers have reported (2) that when maintained in a similar contaminated environment, 67 percent of the progeny of scrapie-affected ewes developed scrapie by 4½ years of age and only 5 percent of the progeny of scrapie-free ewes did so. In this country the pedigrees of affected sheep are carefully studied and bloodlines traced. The frequently recurring relationships observed in these studies seem too striking to be entirely coincidental. One must keep in mind, however, that similar apparent relationships might be observed on the basis of genetically acquired susceptibility or resistance with the susceptible animals being in an environment in which they are exposed to the pathogen. Based on knowledge presented during the seminar, the latter seems to be a valid assumption. Some uninoculated offspring (both sheep and mice) of inoculated parents developed the disease. These, however, were also maintained in a contaminated environment.

Importance of Contact or Lateral Transmission

Although the theory that scrapie is spread from parent to offspring has many advocates, it does not

fully explain the spread in certain flocks. As Gordon (3) has pointed out neither does it elucidate the means by which the disease spreads from one purebred breed to another. Examples are the two Canadian flocks in which a Hampshire and a Southdown sheep, as well as the Suffolks in these flocks, developed scrapie and in Wales where the disease appeared in purebred Welsh Mountain sheep (a breed believed free of the disease) following use of a Suffolk ram on a commercial portion of the flock. Animals other than his progeny came down with the disease. There may, of course, be instances of certain pure breeds being crossed with others; however, the characteristic appearance or performance of some breeds make their crossbreeding unlikely. There is experimental evidence that contact spread occurs in sheep, goats, and mice. The evidence is quite clear; however, in numerous instances investigators have failed to demonstrate spread by contact. One, then, is inclined to believe that contact or lateral spread does occur in the field but may be of less importance in the epidemiology of scrapie than vertical spread from sire or dam with emphasis placed on the role of the latter.

Research studies at Moredun Institute indicate that a likely means of scrapie spread is from affected parent, particularly dam, to lamb. Parry (1, 4) believes strongly that the affected ram is also responsible for scrapie affecting the progeny. Our field experience in this country is that when half sisters or half brothers are affected, the common denominator is usually the ram.

In listing all sheep which are known to have had scrapie in this country and identifying their common sires and dams we find that there were many more sires which had two or more affected progeny than ewes that did so. This may be understandable, at least from a statistical standpoint, as rams have many more immediate progeny than ewes—perhaps on the order of a 25 to 50 to one ratio.

Table II lists 35, apparently normal to the best of our knowledge, rams which each sired two or more sheep, or a total of 116, which were found to have scrapie. One of them a British ram with Code No. 1 is known to have had 18 affected ewe progeny in California and Illinois. The remaining 122 affected sheep, out of our total of 229 in this country, were by 122 different rams not known to have had scrapie. We estimated that these 122 rams had sired an additional 26,000 lambs which are not known to have had the disease. This estimate is on a basis of 50 progeny per ram per breeding year. These data do not fit the research data, otherwise a much larger number of the 26,000 progeny

TABLE II

Normal Rams Siring More Than One Scrapie Affected Progeny

Normal ram code No.	Number affected progeny sired	Normal ram code No.	Number affected progeny sired
1	E E E E E E E E E E E E E E E E E E E E	18	\wedge T E E R R
2	E R	19	\wedge T T=Twins E E R R
3	R R	20	R R
4	E* E* E* R* R* R	21	E E E
5	E* R* R	22	E E E R
6	E E E	23	E E
7	E* E* E E	24	E E R
8	E E R	25	E E E E R
9	E R	26	E E
10	R R	27	E R
11	R R	28	R R
12	E R	29	E E E
13	E R	30	E E
14	E E	31	E R
15	E E R R R R R	32	E R
16	E E E E E	33	E E
17	E E E	34	E E
		35	R R

35 normal rams sired 116 affected progeny (*9 Canadian).
122 normal rams sired 122 single affected progeny and perhaps an additional 26,000 progeny not known to have had scrapie.

1 suspect ram considered normal in this table sired 2 affected progeny.

The 63 affected rams in this country have sired perhaps 7,500 progeny, none of which are known to have had scrapie.

R=37 ram progeny, E=79 ewe progeny.

should have eventually developed scrapie. We wonder why they didn't and submit that the reason is that, again based on the research findings, many more would have developed scrapie had not the eradication program eliminated them before they did so.

Note that two of the affected progeny were by a ram who was a possible scrapie suspect on the basis of his history. He wasn't considered as affected in this table as he died without a diagnosis being made.

The 63 known affected rams in this country have sired an estimated 7,500 lambs, none of which are known to have had scrapie. Again this is quite surprising, if an affected ram plays a significant genetic role in the transmission of scrapie, unless the eradication program has been quite useful in preventing these cases from occurring. Otherwise the research figures suggest that perhaps 25 percent of the 7,500 progeny of

these affected rams could well have developed the disease. Lastly—an important program point before leaving this table—is that if one identifies all half brothers and half sisters of known affected sheep in this country, largely by way of the sire of affected animals, he has located approximately 50 percent of all affected animals. This is an important program item because to ignore such animals is to get into serious difficulty.

TABLE III

Normal Ewes Lambing More Than One Scrapie Affected Progeny

Normal ewe code	Number affected progeny lambled
A	E E
B	E R
C	E R (Twin)
D	E E (Twin)
E	E E
F	E R

6 ewes lambled 12 affected progeny (9 ewe progeny, 3 ram progeny).

213 normal ewes lambled 213 single affected progeny and perhaps an additional 1,000 progeny not known to have had scrapie.

3 affected ewes (not laboratory confirmed) lambled three affected progeny.

The 166 affected ewes in this country have perhaps lambled 325 progeny—only one of which is known to have had scrapie.

Table III is similar to table II except that it applies to dams rather than sires of affected sheep.

Considering the total of 229 affected sheep, in only six instances did two affected sheep share the same dam. In two of the six instances the affected sheep were twins. In all other instances different sires were involved.

The remaining 217 affected sheep of the total of 229 were progeny out of 217 different ewes. It is estimated that these 217 ewes had some 1,000 progeny in addition to those found to have scrapie. None of the additional 1,000 progeny are known to have had the disease.

Now of the 217 other dams 1 was affected (confirmed) and 3 others believed affected but brain tissues were not taken for laboratory study. Actually, the 166 known affected ewes have lambled perhaps 325 progeny, only 4 of which are known to have had scrapie, 3 of these were laboratory confirmed. That doesn't say a greater number didn't have it. But if they did, we didn't know about it.

Now we have to take into account here—and I'm sure if I didn't say this the statisticians would remind me of it—that we don't really know how many of these were actually at risk. Some of them were eliminated for various causes or died before they were old enough to show signs of the disease. Again I think this speaks well for the eradication program. Because, if we are to accept the research information as presented, it would certainly suggest that a considerably greater number remaining, perhaps as many as 67 percent of these 325 progeny of affected ewes, should have taken scrapie had they not been eliminated by the program.

Figure 3 shows affected progeny of 12 normal rams out of 33 different dams and reflects the situations in California and in Illinois as seen during the early scrapie outbreaks.

In the 2 associated California flocks—considered one outbreak—20 ewes and 1 ram were found to have

scrapie. Seven of them were the immediate progeny of British (imported into Canada) ram, Ram No. 1. Their dams had been imported into California as bred ewes. Ram No. 6 sired three of the affected ewes. Ram No. 17 sired three, seven different rams sired one affected ewe each and one ram, No. 107, sired the one affected ram.

These seven affected ewes sired by Ram No. 1 were from 36 to 45 months of age. Of the remaining 14 affected sheep, 6 were born on the premises and were 32 to 44 months of age. Eight had been purchased from other breeders. Two of the latter had been on the infected premises 24 months, the remaining six, 4 to 5 years. One of the eight purchased sheep was 44 months of age, the other seven were 66 to 93 months old.

It does seem significant that with the exception of one all the purchased sheep were considerably older than those born on the premises and all but two of the

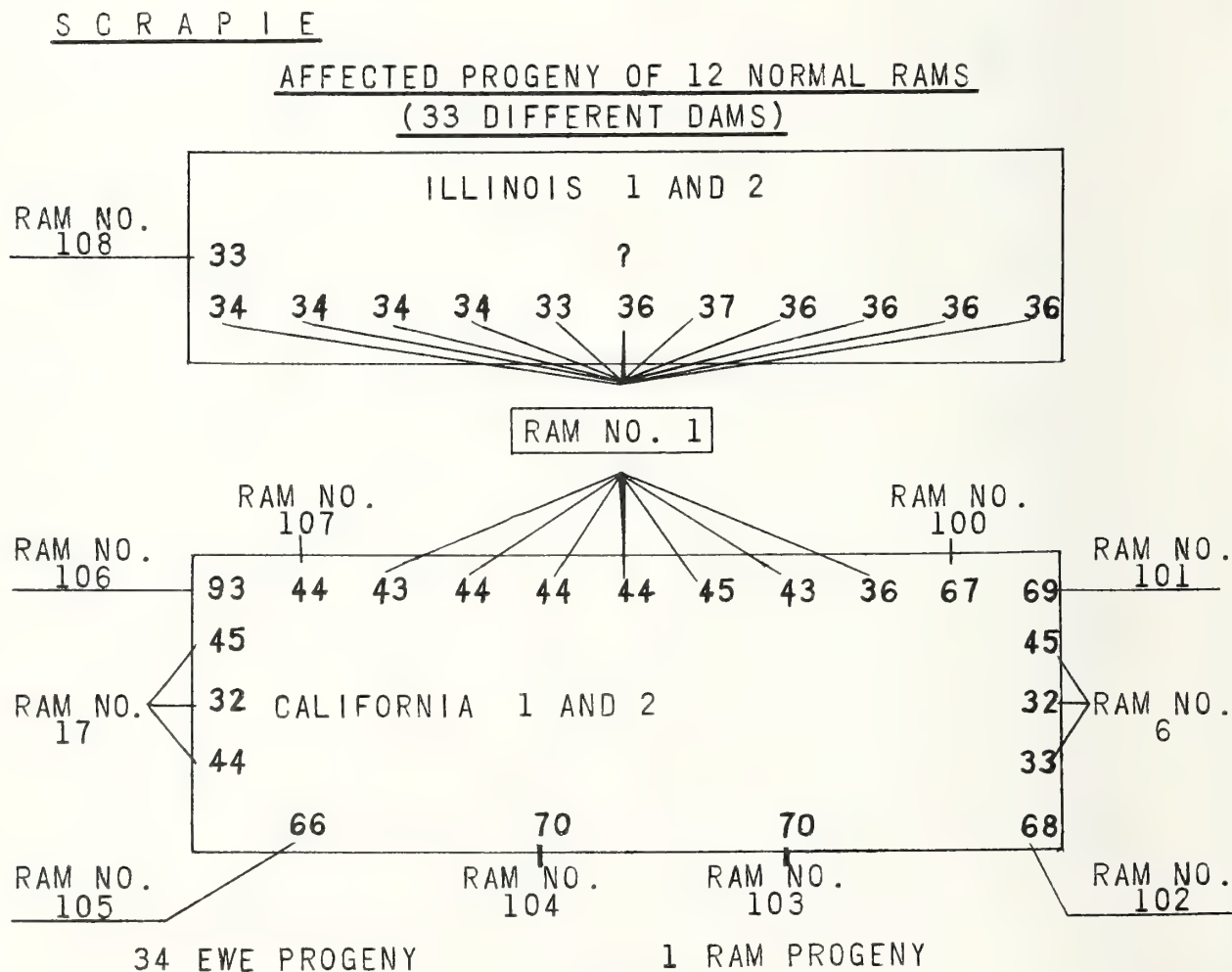


FIGURE 3

purchased sheep had been on the infected premises from 4 to 5 years.

All 21 affected sheep were out of different dams. One of these dams was considered affected but was not laboratory confirmed. If one assumes that the ewe plays a very important role in the appearance of scrapie in her immediate progeny, it does seem rather a rare coincidence that these 21 ewes—which were actually bred by 14 different breeders—should at this one time and one place and each separately and in her own right give simultaneous evidence of this phenomena.

It would seem that a more realistic hypothesis might be that a pathogen was involved and the premises contaminated with this pathogen. One must also note that the extended pedigrees of these affected sheep, and I am sure other sheep on the premises as well,

included ancestors that one would be dubious about. This is also true of many sheep of this breed in this country.

In the Illinois outbreak—actually two flocks—the same ram, Ram No. 1 sired all except two of the affected sheep and those two were in fact purchased out of the same Canadian flock where Ram No. 1 was located and eventually died. All of the 12 affected sheep had been purchased out of this flock and imported.

Again the 13 affected sheep were out of 13 different ewes from 4 different breeders.

Figure 4, showing affected progeny of five normal rams and six different ewes, depicts a somewhat different picture. Six ewes in this flock were found to have scrapie. Two were by Ram No. 23 and each of

SCRAPIE

AFFECTED PROGENY OF 5 NORMAL RAMS (6 DIFFERENT EWES)

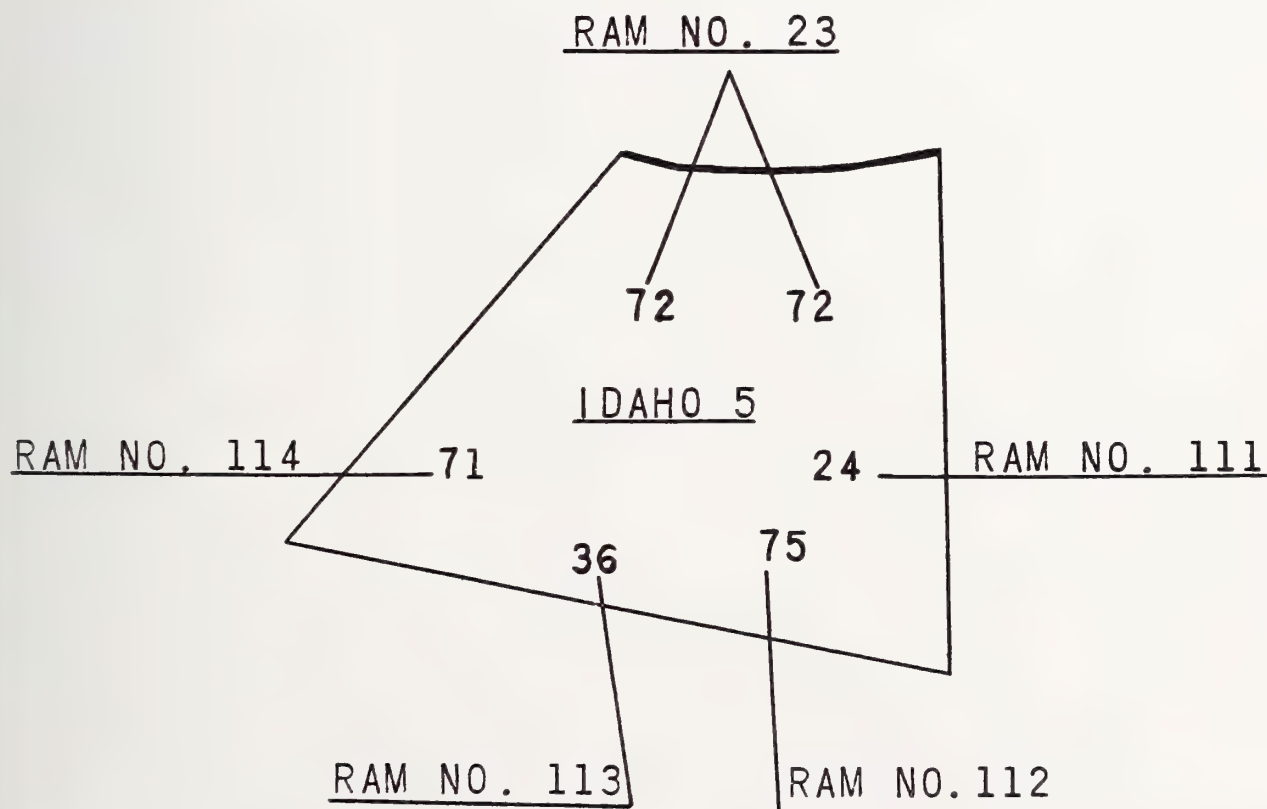


FIGURE 4

the others were by different rams. All affected ewes were out of different dams. The six dams were from five different breeders and the five sires were from four different breeders.

Three of the six affected ewes had been bred in the infected flock and were 24, 36, and 71 months of age. The other three had been purchased out of other flocks and were 72, 72, and 75 months of age and the two for which records are available had been on the infected premises for 5 years.

There were some 1,700 sheep in this flock with perhaps 500 in the scrapie age group. There were 6 known affected sheep involving perhaps 10 different sheep families.

In summing up figures 3 and 4, if we assume in instances such as these that only vertical spread of scrapie occurs, we must then further assume that the individual affected sheep shown in the figures were destined to come down with scrapie wherever they might be. We must also assume that the numerous sheep families represented by these individual affected sheep were spreading the disease because of their particular blood lines. This would mean that in this country we must be having many hundred of cases of scrapie. Evidently this is not the case and one must again turn to the hypothesis that a pathogen and a contaminated environment are involved.

SUMMARY

The 138 outbreaks in the United States illustrate the potential danger of an insidious disease like scrapie in

a country such as ours where lively trade among breeders can result in wide distribution of sheep and their diseases.

We look forward to additional research results, particularly those designed to explain natural transmission. Precise information in this regard will permit more exact identification of potentially dangerous animals and, it is hoped, provide for effective eradication procedures necessitating the slaughter of fewer sheep. This knowledge is especially important in the absence of a diagnostic test in the live animal.

The chances for ultimate success appear good if we have an assurance of finding most affected animals and continue to follow sound eradication procedures and are supported by ample research.

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Spread of Scrapie from Inoculated to Uninoculated Mice¹

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The general experience with scrapie in our laboratory (1) as well as in the laboratories of others in this country (2) and in Great Britain (3) indicates that inoculated and control mice can be housed in the same room for prolonged periods of time without transfer of the infection from mouse to mouse. There are, however, two recorded occurrences of spread of scrapie from experimentally infected mice to uninoculated control mice. One of these is the report by Pattison (4) on occurrence of scrapie in 15 of 49 mice held in direct physical contact for 8 to 14 months with successive batches of scrapie inoculated mice; the other report is that of Dickinson, Mackay, and Zlotnik (5) on the appearance of scrapie in 2 of an unprescribed number, but less than 24, uninoculated mice housed in the same cages for 11 and 14 months with a series of other mice with experimentally induced scrapie. In Pattison's work, fighting occurred between inoculated and uninoculated animals; because of this Pattison suggested that scrapie might have spread to uninoculated mice by ingestion of tissue fragments containing the scrapie agent. In Dickinson's study it was reported that one of the pair of scrapie affected control mice was bitten 9 months before onset of its scrapie illness by an experimentally infected cagemate, but for the other mouse there was no evidence for either fighting or for cannibalism.

It is the purpose of this note to report observations made in our laboratory over a 3½ year period which suggests that scrapie, after its experimental introduction into mice, might become a contagious infection of low order for this animal.

¹ Following its presentation at the symposium this paper was subsequently published in the Proceedings of the Society for Experimental Biology and Medicine, Volume 120, Number 1, pp. 108-110, 1965.

MATERIALS AND METHODS

Mice employed in this work were of 5 breeds: Swiss GP, Swiss NIH, CFW, CDF₁, and C₅₇ black. All mice were obtained from the Animal Production Unit, National Institutes of Health. The breeding history of each strain is known. The occurrence of scrapie-like illness and scrapie-like lesions in uninoculated mice at the National Institutes of Health has not been recorded. For our purposes here the 5 breeds are not differentiated.

The mice were housed in plastic cages of the shoebox type fitted with perforated metal lids. Seventy cages, each holding 5 mice, were kept on a single rack. Mice inoculated with scrapie materials and uninoculated control mice were housed in separate cages on the same rack. An effort was made to keep control mice on the upper shelves and inoculated mice on the lower ones. Bedding in the cages was changed once and sometimes twice a week. About once a month the plastic cages were washed in water at 180° C. No effort was made to return the mice to the same cage from which they were removed. Mice were handled with forceps which were periodically disinfected by dipping in saponified cresylic acid.

The scrapie material employed was from three sources: brain from an experimentally infected goat obtained from Mr. Pattison, Agriculture Experiment Station, Compton, Berkshire, and two brains from naturally infected sheep obtained from workers in the U.S. Department of Agriculture. Inoculation procedures were carried out as previously described (1).

Diagnosis of scrapie in all affected mice was made on the basis of clinical observation. In addition, microscopic examination was made of the brain and cord of a small number of scrapie affected mice.

RESULTS

Brain material from the experimentally infected scrapie goat was inoculated into 200 mice in August 1961 (1). One hundred uninoculated mice were maintained as shelf controls. The infectious material used represented the ninth passage of the scrapie agent in goat brain. It induced in the inoculated mice a central nervous system disorder associated with clinical scrapie signs which have already been described by Chandler (3) and by us (1). The incubation period ranged from 7 to 13 months and was dose dependent. By October 1962 or 14 months after the beginning of the experiment 139 mice had died with scrapie signs, 28 had died of nonspecific causes and 33 had been killed for harvest of tissue and serum.

In October 1962, brain material from a naturally infected sheep was inoculated into 100 mice. Inoculation of a 10 percent saline suspension of this material was followed by development of scrapie illness in the test mice with incubation periods ranging from 9 to 13 months. By the end of the 15th month in this group of 100 mice, 74 had died with scrapie signs, 19 had died from nonspecific causes and 7 had been killed for tissue and serum. Passage of brain material from one of the affected mice in January 1964 to 100 other mice was followed at approximately 5 months by development of scrapie illness in 89 of the passage mice. The remaining 11 mice were killed for tissue or serum harvest or died of nonspecific causes.

Finally, in November 1962, brain material from another naturally infected sheep was inoculated into 150 mice. At this time 100 uninoculated shelf controls were established. Inoculation of the material was followed in the test mice at approximately 1 year by development of scrapie illness. Seventy of the 150 mice died with scrapie signs, 20 died of nonspecific causes and the remainder are still alive or have been sacrificed for study purposes.

In uninoculated mice scrapie illness was first observed in the animals set up to control the goat brain study. In February and March 1963, or 18 and 19 months after scrapie was introduced into our laboratory and approximately 6 months after the last scrapie death had been recorded for the mice inoculated with the infected goat brain, three control mice exhibited scrapie illness. Brains of 2 of the 3 control mice were examined microscopically and the lesions were consistent with those found in brains of mice inoculated with the goat brain material. Unfortunately because of other work, it was not possible to hold these control mice for a longer period of time and they were discarded in April 1963. However, mice inoculated with

sheep brain material together with their control mice were kept under prolonged observation. Of the 100 mice set up in November 1962 to control the sheep brain study, 4 developed scrapie illness in the 2 years they have been held for observation. Clinical evidence of scrapie occurred in these mice 16 to 22 months after initial exposure. Brain and cord from one of the mice in this group have been examined microscopically and the lesions are consistent with those of scrapie.

DISCUSSION

It seems highly probable that scrapie, after experimental introduction into mice housed under conditions of our work, might become a contagious agent for this animal. If this is the case, precise experimentation with this agent cannot be done in the absence of strict isolation facilities. We now have studies underway to determine whether or not the precautions taken by Rowe and his coworkers (6) and by us to prevent spread of viral hepatitis in mice are applicable in prevention of spread of scrapie. This involves use of Trexler isolators and jars with fiberglass filters in the lids (6). It seems likely that virus might have been carried from cage to cage by forceps, scattered bedding, or unwashed hands, mixed water bottles or cages, or even insufficiently sterilized cages in the long period of over 1 year of daily handling and feeding of the animals. Thus, airborne infection, while not ruled out, would appear to be unlikely in view of the small number of control mice who have acquired scrapie. It is established that scrapie is readily transmissible to sheep, goats, and mice by feeding (4, 7).

Pattison (4), in interpretation of his findings in mice, emphasized the necessity for caution in extrapolating experimental data obtained with one species in terms of another. He has presented convincing evidence that under the condition of his experiments, scrapie did not spread after prolonged and intimate contact from sheep to sheep or from sheep to goats. Our prime concern, of course, is the behavior of man following prolonged exposure in the laboratory to the scrapie agent. There is no information as to whether or not human will react to contact with scrapie agent as do sheep and goats or in a manner similar to the behavior of mice, or in a manner like none of these hosts. We have inoculated 4 rhesus monkeys both subcutaneously and intravenously with the mouse scrapie agent (10 percent brain in buffered saline) and to date, after 8 months of observation, no symptoms of disease have yet appeared in the monkeys. It seems appropriate to emphasize that precautions employed to prevent

spread of conventional viral agents to man and mice might not be sufficient in work with scrapie. In this connection the still unverified observations of Pálsson, Pattison, and Field (8) of the emergence of a scrapie-like condition in sheep inoculated with brain material from an acute case of multiple sclerosis demands attention. Either there is more spontaneous scrapie in "control" sheep than the investigators suspect, or the multiple sclerosis brain material has provoked or activated a scrapie-like process.

The total experience with scrapie emphasizes the urgent need to develop a specific serologic test for scrapie. Such a test will not only find wide use in veterinary medicine but might also be of help in determining whether or not scrapie is of potential or real danger to man.

SUMMARY

Scrapie occurred in 7 of 200 uninoculated control mice housed in the same room with, but caged separately from, successive groups of scrapie inoculated mice. Clinical evidence of the disease appeared in controls as early as 16 months and as late as 22 months after initial exposure to scrapie inoculated mice. The significance of the observations is discussed.

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DISCUSSION

ZLOTNIK: We had another case where one of our scientific workers inoculated some mice and, after a while, found that some of the marks on the ears were obliterated and so he decided to repunch them all. Somewhat later scrapie began to appear in some of the controls and it looked as if the disease had been transmitted, by punching the ears, from those that had been inoculated with scrapie, say about 6 weeks before. I would like also to recall a paper by the late Dr. Greig who proved (I think, in view of what we know now, beyond doubt) that scrapie can be contracted from the environment; he obtained infectious sheep from infected pastures without any direct contact from an infected animal. One of these cases was the cause of the breakdown in the louping-ill vaccine that gave rise to cases of scrapie in Australia and New Zealand.

FIELD: May I ask what the workshop feels with regard to the mouse work that is being done at the moment? Those of us that do not have the excellent facilities for isolating cages and taking the precautions that Dr. Morris has suggested may feel a little uneasy now.

MORRIS: It is not the purpose of this presentation to put a damper on the amount of work that is being done. We have not lessened ours, but have merely taken a few extra precautions. The overwhelming experience of all the scrapie workers is that spread of the disease from inoculated to uninoculated mice occurs only rarely.

CASALS: The question involved is really how much one can depend on holding animals for a long time and whether or not they pick scrapie up from their cage mates or from what you inoculated. Well, if only 3 out of 100 pick it up over a period of several years, and you have a titration in which 90 or 95 percent of the mice come down, you would accept that those are due to your inoculation; and, besides, if between the secondary and primary cases you have a gap of about a year, it seems to me that you can pay attention to your results without being too unduly alarmed about these cases of contamination.

HOTCHIN: I would like to make a comment on this business of cross infection. We are worried about it with LCM and we have now experience with three different animals, the mouse, the human, and the guinea pig. When we put mice in some of our rooms where we have chronically-infected other mice, and leave these normal mice there for a year

or so we have found no evidence of any cross infection from cage to cage as judged by tests for virus immunity. In regard to humans we have had three cases which were apparently picked up by personnel checking mice, probably by shaking the cage as a lazy way of looking for carcasses, thereby taking in a good breath of contaminated dust. So we can conclude that (natural) LCM infection is not confined to mice. Moreover, if we put guinea pigs in the infected mouse rooms, they practically invariably come down with LCM in about 2 weeks, wherever they are placed in the room, so we conclude that the virus is present everywhere in the air in the infected mouse rooms in insufficient quantity to infect mice to a noticeable degree, but enough to infect some humans and almost all guinea pigs.

EDDY: I would like to mention another virus. In working with polyoma virus a few years ago, there were people who could not induce tumors in their animals, the reason being that the animals were carrying this virus in an inapparent form and they apparently had enough antibody so that tumors could not be produced in them. You are working with scrapie, which is a very stable virus, and one can assume that it is not going to be destroyed as easily as influenza, for example, which is rapidly destroyed in the air. It may be that the animals which show no scrapie still have an inapparent infection.

MORRIS: It might be wise in this work to do always as we have done in most instances, that is, keep the control mice for extended periods after the inoculated mice have died.

The Virology, Pathology and Epizootiology of Aleutian Disease in Mink

Chairman

J. HOTCHIN

Some Observations on the Natural Occurrence of Aleutian Disease¹

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The epizootiology of Aleutian disease (AD) is an enigma to the mink rancher and the researcher alike. At the outset, we must confess that we do not know the primary means by which the disease is spread among ranch mink. It appears that both vertical and horizontal transmission occur. Furthermore, the durations of the prodromal stage and the infectious period are unknown.

A historical survey of AD sheds considerable light on the rapidity of its spread throughout the United States and foreign countries. Prior to 1940, in the early years of the mink industry, the ranches were small and relatively inefficient. Mink were trapped in the area surrounding the ranch or occasionally were brought from as far away as Alaska or Labrador. They were considered to be what is termed a "standard dark" or "wild type" mink. The problems associated with a proper diet and the devastating outbreaks of distemper and botulism were the ranchers' main concern. Thus, one cannot say for certain whether an insidious disease such as AD was present in these first ranch-raised dark mink. However, if it was present, it smoldered along unnoticed or perhaps was confused with some other malady.

The first ranchers, not realizing the possibility of commercially important mutations, promptly disposed

of "off-colored" mink. In 1941, an astute rancher near Astoria, Oreg., saved for breeding a gun metal colored mink that was noticed in a litter of standard dark mink.

The color phase was called "Aleutian" after the Aleutian fox which has a similar pelt color. The gene, which was inherited as an autosomal recessive trait, was designated by the symbol "a". By crossing Aleutian mink with other mutant mink, several sub-strains were developed which were collectively called "blue mink." Since the pelts were desirable colors, the mink were extremely valuable and commanded high prices. There was a brisk sale of breeding stock and most of the industry geared itself for production of these mutants. In the early forties, Aleutian mink were sold to ranches in various parts of the country. Soon ranchers began reporting losses due to a condition seemingly confined to "blue mink."

Mink ranchers first noticed the condition when their "blue mink" lost weight, although there was usually no noticeable drop in food consumption. Faulty digestion was manifested by cereals and/or other undigested food stuffs in the feces. Dark tarry droppings were occasionally observed. Affected mink exhibited polydipsia. About 10 to 20 percent of those visibly sick bled at the mouth. Ragged ulcers at the gingival border, usually involving the teeth, bled when the mink were handled. Ranchers called them "bleeders." The mink were anemic as evidenced by pale mucous membranes and foot pads. They became progressively thinner until death intervened.

Hartsough (1) first observed the gross lesions of AD in 1946. These lesions were recorded in "blue mink" shipped to the Midwest. At first the disease was thought to be limited to the *aa* genotype inasmuch as

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AA and *Aa* mink raised on the same ranch appeared resistant. Some ranchers tried to cope with the malady by crossing back their Aleutian mink to *AA* stock and/or breeding *Aa* and *Aa*. While the *aa* progeny was somewhat stronger than their original Aleutian breeding stock, they were still more responsive to the disease than *Aa* or *AA* mink.

As knowledge of the malady increased, it became known as "kidney disease." Depending on the stage of the disease, the kidneys were enlarged and reddened, or swollen, pale and pitted. Other necropsy findings included initial enlargement of the spleen and lymph nodes, and occasional yellowish brown mottling of the liver.

The Infectious and Contagious Nature

The losses from AD increased each year. Then a series of "vaccine incidents" occurred, which focused attention on the malady, suggesting that the disease might be of an infectious nature (2). Prior to the advent of chicken-embryo-origin attenuated distemper vaccines, it was common practice to prepare inactivated autogenous tissue vaccines. To make this vaccine, spleens were collected from distemper infected mink and ground with saline containing 0.3 to 0.5 percent formalin. After the mixture was incubated for a variable length of time—often dependent on how badly the vaccine was needed—it was injected. Following the use of these vaccines, widespread AD losses occurred. In 1949 one rancher lost 500 mink to AD after vaccination for distemper with such a vaccine. Floyd Marsh, a truly colorful mink raiser, said "they didn't make baskets big enough to pack out my dead mink."

A few years later a Connecticut mink rancher who had AD on his ranch decided to try one of these homemade vaccines against the disease. The result was reminiscent of the scrapie outbreaks which followed the use of louping-ill vaccine (3). Helmboldt and Jung-herr (4) reported that nearly all of the inoculated mink were dead of AD after 6 months!

By 1950, it was apparent to many mink ranchers that AD was contagious. When mink from an affected ranch were brought to a "clean ranch," the disease appeared in the purchased breeding stock as well as in other mink on the ranch. Such a prospective study was made in western Oregon in 1955 by our coworker, Dr. Keith Farrell. The rancher had many desirable mutations which, although he did not know it, were infected with AD. They were sold to several other ranches and new foci of disease appeared on these

ranches. Overseas shipments by many U.S. ranchers of infected mink may have spread the disease to Scandinavia, England, Japan, Canada, West Germany, and elsewhere. However, the possibility exists that Aleutian disease was present but not recorded in these countries prior to the U.S. mink exports.

With the history of Aleutian disease in mind, we might speculate about the apparently sudden appearance of this "virus." Two alternatives appear possible: (1) the virus appeared about the same time and in the same area as the Aleutian gene mutation, or the virus might be associated with the Aleutian gene; (2) the virus was present in mink prior to selection of the Aleutian mutant but was not recognized until sufficient numbers of highly responsive *aa* mink became available in the ranch mink population. In considering the first possibility, it appears extremely unlikely that matching mutations would occur, not only at the same time, but in the same area.

The second proposal is not only more probable but is intellectually more acceptable. When the "blue mink" were shipped from the Oregon ranches, some were infected with AD while others were free of the disease. Infected animals established new foci of disease on clean ranches. On the other hand, when noninfected "blue mink" were sent to ranches where Aleutian disease was present in standard dark herds, the newly purchased "blue mink" served as sentinel animals by succumbing to AD.

There is no direct proof to support either of these possible explanations for the origin of the AD agent. However, if we could locate preserved tissues or tissue sections of standard dark mink with AD lesions in the late thirties, it would prove the latter possibility.

The Chediak-Higashi Syndrome

It was not until 1963 that a possible explanation for the remarkable genotype responsiveness of *aa* mink to AD virus was recorded. Leader, et al. (5) and Padgett, et al. (6) described a condition occurring in mink and cattle which resembled strikingly the Chediak-Higashi syndrome (CH-S) previously described in man (6).

In man, mink, and cattle, the syndrome appears to be caused by a simple recessive non-sex-linked gene. Among mink, the only animals affected with this condition are those which are homozygous recessive for the Aleutian gene "*a*." Thousands of mink have been examined, and we have never observed a mink with the *aa* genotype which does not have the CH-S.

TABLE I.—Losses From Naturally Occurring AD on One Ranch Comparing *aa* and *Aa* or *AA* Mink From December 1960 to December 1961

	Aleutian mink <i>aa</i>			Non-Aleutian mink <i>Aa</i> or <i>AA</i>		
	Total number of animals	Total death loss	Mortality rate (percent)	Total number of animals	Total death loss	Mortality rate (percent)
Adult females (animals carried over for breeding)	655	311	47.5	740	44	5.9
Adult male (animals carried over for breeding)	164	128	78	185	18	9.7
Kittens (born during current year)	1,800	162	9	2,130	38	1.8

In one study of CH-S in mink, Padgett, et al. (7) inoculated 40 *aa* and 40 *Aa* or *AA* mink with 10^4 ID₅₀ of the AD virus. All animals developed Aleutian disease. Of those with the *aa* genotype, all but 4 were dead within four months after inoculation. In the same period, only 4 of the non-Aleutian (*Aa* or *AA*) animals were dead. After nine months, one-half of the latter group survived and 6 mink lived for more than a year. These findings and the results of similar work suggest that all mink are susceptible to AD, but early death occurs primarily in mink of the Aleutian genotype. Indeed, it was for this reason the name Aleutian disease was given to the condition. The difference in mortality rate between Aleutian and non-Aleutian mink in naturally occurring AD is illustrated in table I, and will be discussed under the section on mortality rate.

The idea that the genetic constitution of the host has a profound effect on the severity of disease caused by a particular virus is certainly not new. One strain of potato X virus kills one variety of potato, causes a mosaic in another and is completely asymptomatic in a third. All three varieties of potato are capable of transmitting the virus.

Seasonal and Age Incidence

The yearly loss pattern is related (1) to the age of the mink, and (2) to seasonal stress factors. To understand this pattern, a brief explanation of the yearly ranch operation is necessary (fig. 1). Mink are bred in March, generally whelp in May and the young kits are weaned in June or July. In late July and August, the litters are split to prevent fighting and subsequent pelt damage. At this time, the young mink (kits) are put into individual cages which vary somewhat in size on different ranches—generally about 12 x 18 x 24 inches. The cages are made of 1/2-inch galvanized wire

mesh and usually contain a wooden nest box. They are placed about 2 inches apart and 18 inches off the ground. Both kits and adults are pelted in November or December at about 6 or 7 months of age or are held as breeding stock for the following year. Occasionally, productive healthy adult females are held for a second breeding season.

In adult-breeder female mink of any genotype, the first loss peak usually occurs in May during whelping (fig. 2). Losses occur in the early fall in both kittens and adults when they are subject to the stress of molting or during the first cold weather or sudden temperature changes. For example, in a herd of 10,000 mink where there is a fair amount of AD infection, 40 to 50 mink may succumb with an abrupt change in the weather. Failure of an automatic water-

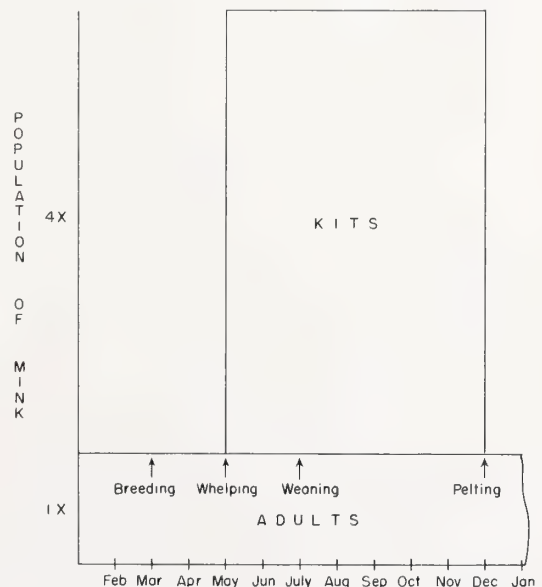


FIGURE 1.—The relative population of mink on a ranch through 1 year.

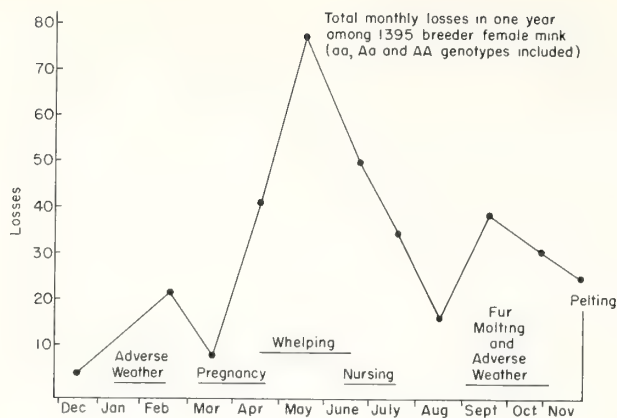


FIGURE 2

ing system in freezing weather will also result in a sharp loss peak due to the stress on the mink.

While adult losses occur usually in chronically infected animals in the terminal stages of the disease, fall losses following a short rapid course are observed in primarily *aa* animals. Losses also occur among *Aa* and *AA* kits, but it is rare to find heavy losses in these groups. *Aa* and *AA* infected animals frequently survive to pelting showing no sign of the disease.

Transmission

Vertical Transmission

The familial occurrence of deaths from AD was first recognized by ranchers who raised some of the early Aleutian and sapphire mink. Ranchers reported various patterns of AD deaths (fig. 3) but, in general, when the female was infected, it appeared more likely that her litter would contain infected kittens. If we

assume that the kits are susceptible in the period prior to weaning and separation, it appears that if a dam is infected, there would be sufficient direct contact with her young in the confinement of a nest box to effect transmission. When the iodine agglutination test (I.A.T.) (8) became available, it seemed profitable to study the prevalence of the disease within families at pelting.

Henson, et al. (9), studied the problem using the I.A.T. and confirmed the ranchers' belief of the importance of the affected dam. Thirty-one families of mink were tested on a ranch having a large number of AD deaths. Thirty-two of 71 kits (45 percent) from 14 positive dams were positive to the I.A.T. Conversely, 15 kits of 78 (19 percent) from 17 negative dams were positive. Thus, vertical transmission from the dam to part of her litter must be considered.

Horizontal Transmission

Current ranch studies conducted at this laboratory (7) have revealed that horizontal transmission between families also occurs. Before and after separation, 145 families of mink with a total of 680 kits were examined at monthly intervals using the I.A.T. In this study, it was shown repeatedly that animals found to be negative by the I.A.T. for several months, which were housed in cages next to positive animals, converted to positive. In addition, on several occasions, the infection was traced from cage to cage, as far as four cages (64 inches) away from the original source of infection. While there may be as yet unrecognized explanations for this phenomenon, it does suggest horizontal transmission of Aleutian disease between families.

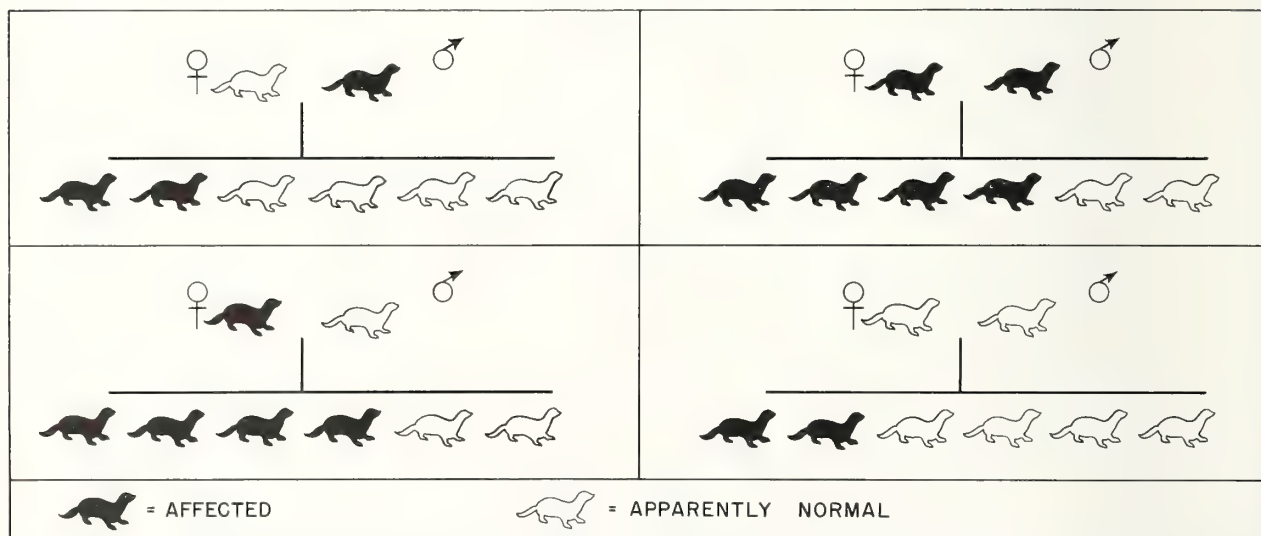


FIGURE 3.—The distribution of naturally occurring Aleutian disease among selected families.

Infectious Materials and Natural Transmission

The first solid evidence of a natural transmission mechanism was reported by Kenyon, et al. (10). In this study, urine was found to contain the virus. We confirmed this work and found virus in the feces as well (11). However, we could not exclude fecal contamination of urine or contamination of feces. In any event, it appears that the virus is present in the feces and/or urine. In addition to feces and urine, our study revealed that blood, serum, saliva, and bone marrow contained the agent, and of the materials tested, only colostrum milk was apparently free. However, since only four animals were used in testing the last fluid, a larger trial involving more animals is needed before valid conclusions can be drawn.

The results of our efforts to learn of the natural routes or transmission are shown in table II. In this experiment, mink were also infected by oral and aerosol routes. It would appear further that infected spleen was more infective than feces; however, no attempt was made to quantitate the virus in the inocula.

The lack of an *in vitro* system for the detection of virus is a serious detriment to research on Aleutian disease. At present all tests for the presence of virus must be done in susceptible mink, which is an expensive procedure.

Infectious Period

The infectious period of AD appears to be of long duration. Kenyon (10) demonstrated the virus in the urine of an advanced case of AD. We have scanty

evidence that virus was demonstrated in the serum of a mink 2 years after experimental infection. In this study, one of two test mink was infected. Presently, a trial which will offer more useful data is in progress.

Case Fatality and Mortality Rates

If one considers AD in the context of ranch operations, it is impossible to calculate a case fatality rate, since the population of mink on all ranches is decimated at pelting time in November and December. Naturally, ranchers select for pelting those animals that are sick, unthrifty or have poor pelt quality and color. This accounts for low December and January losses when examining seasonal death loss patterns. They keep for breeding animals that are apparently healthy and of good size and color. Pelting precludes the calculation of the case fatality rate. It is commonly accepted that AD infected mink invariably succumb. This may well be valid when considering *aa* mink. On the other hand, there is now limited evidence that *Aa* or *AA* mink may be infected with Aleutian disease and recover if a rise plateauing, and subsequent fall to normal limits in serum gamma globulin is a valid criterion.

The mortality rate is dependent on whether *Aa*, *AA* or *aa* mink are involved. One of our test ranches, having a population composed almost entirely of "blue mink," had an increasing annual death loss until 1961 when it reached 700 deaths among 5,000 mink—a mortality rate of 14 percent. We compared mortality rates of naturally occurring AD in *Aa*, *AA* and *aa* mink on another test ranch in 1960 (table I). The table reveals the mortality rate to be 5- to 8-fold higher among *aa* mink than *Aa* or *AA* mink. The chronicity of the disease is indicated by the marked difference in mortality rate between kits and adults regardless of genotype.

Control

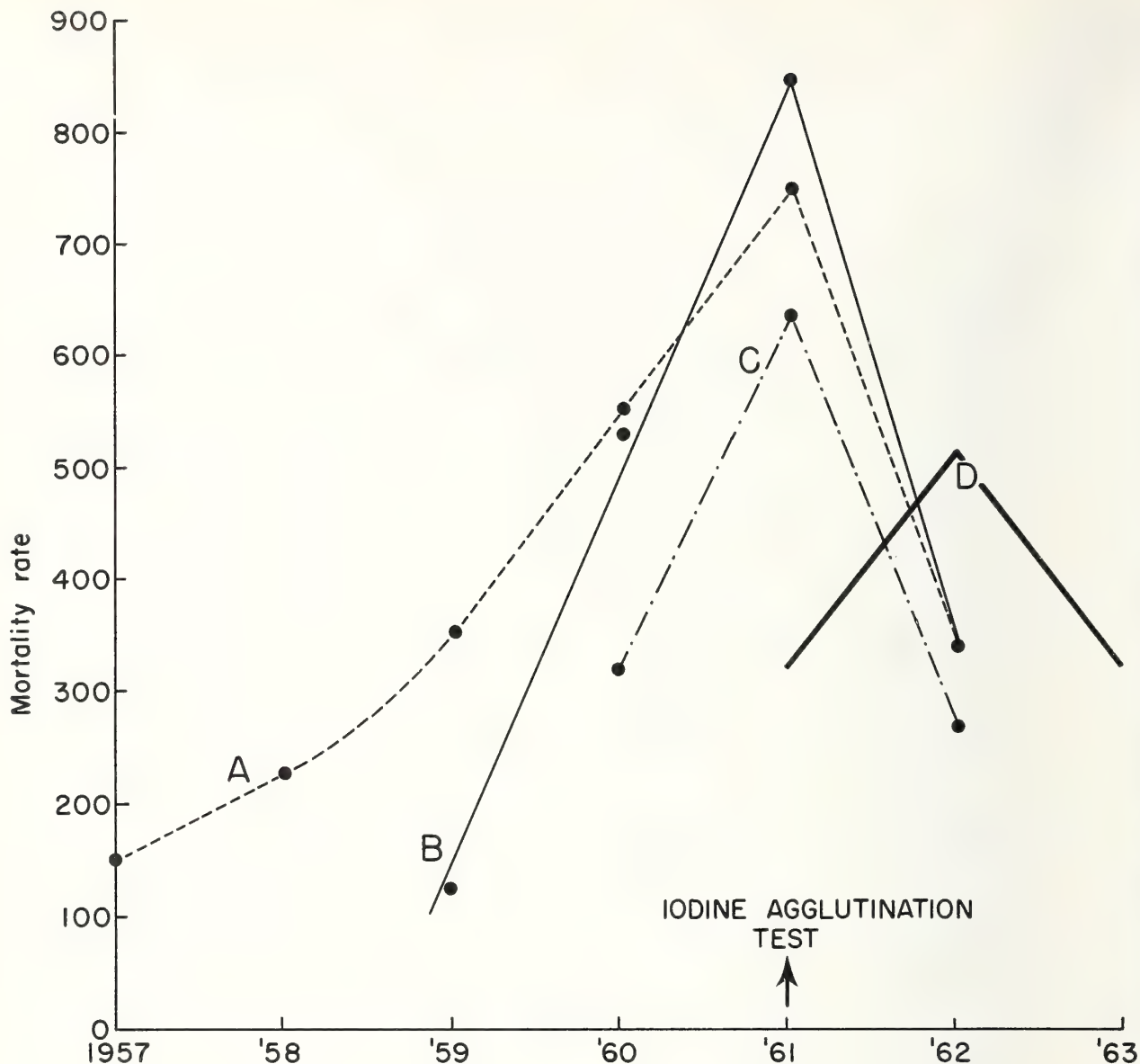
In 1961, Henson and his coworkers reported that AD affected mink exhibit a hypergammaglobulinemia (12). In 1962, the same workers adapted Mällen's iodine test (13) as a useful ranch procedure for the detection of subclinically infected mink (8). In our hands, the I.A.T. becomes positive when the gamma globulin is greater than 2 grams per 100 ml. of serum, and the A/G ratio = 1. Depending on the stage of the disease, the test has an accuracy of 75 to 95 percent. At this writing (May 1965), millions of mink have been tested in all mink-raising areas of the world.

TABLE II.—Material From Advanced Cases of Aleutian Disease Tested for the Presence of Virus by the Inoculation of Susceptible Mink

Source of Inoculum	Results ¹
Whole blood	5/5
Undiluted serum	15/15
Urine	6/9
Colostrum milk	0/4
Feces	5/5
Saliva	5/5
Bone marrow	9/9
Normal mink control ² (no exposure)	0/55

¹ Number of mink infected/number exposed.

² Includes 5 mink which were given 1 ml. inoculations of normal mink serum and 15 mink receiving 1 ml. of nutrient broth.



Total crude mortality rate per 5,000 mink on 4 ranches

FIGURE 4

In figure 4, we plotted the total crude mortality, adjusted to 5,000 mink on 5 different western Washington ranches located within 25 miles of each other. On ranches A, B, and C, annual losses of increasing severity occurred through 1961. In November and December 1961, the iodine test was conducted on all mink on these ranches held over for breeding. Only mink which were I.A.T. negative were kept.

The mink on ranch D were first tested in November and December of the following year (1962).

Therefore, we felt that we could consider ranch D an untested control. The value of the test is documented since the losses on ranch D were ascending during 1962 while the others were dropping. Furthermore, the losses declined in 1963 on ranch D, the year following the initial test.

The relationship of AD to sterility was shown by Olsoni and Kangas (14). They found that of 1,116 sterile females from 32 farms, 496 or 44.4 percent were I.A.T. positive. I.A.T. positive reactions varied on

individual ranches from 8.3 percent to 87.5 percent. The relationship between the I.A.T. results and the number of kittens per female is shown in table III.

At the present time, the iodine agglutination test is the only measure which has proven effective in the control of AD. However, the I.A.T. has disadvantages. There is a period of about 3 weeks after infection before the test becomes positive, and ranchers cannot afford to kill infected animals other than when their pelt is prime.

TABLE III.—Relationship Between I.A.T. Reaction and Number of Kittens of Female Mink

Blood reaction I.A.T.	Number females	Total number kittens	Kittens/female
—	150	614	4.1
+?	15	51	3.4
+	53	166	3.1
2+	40	132	3.3
3+	32	46	1.8
4+	2		
	292	1,009	3.5

These are the results of iodine agglutination tests reported by Drs. Olsoni and Kangas of the State Veterinary Medical Institute, Helsinki, Finland.

Future

Before we can fully understand the natural history of AD, we should (1) confirm horizontal transmission circuits and investigate whether kits can be infected in utero or neonatally; (2) determine the duration of the infectious period; (3) determine if one or more strains of the AD agent exist; (4) study the range of host species involved.

One of the major difficulties which confronts investigators is detection of the AD agent. Presently, the only indicator host is the mink. Before real progress can be made, the agent must be adapted to a sensitive cell line or other convenient laboratory animal. Hopefully, an in vitro system for the detection of antibody will be found—if antibody occurs in this malady. Such

a test would provide relevant information on the antigenic constitution and behavior of the agent in animals.

Aleutian disease will remain an enigma until we can determine the means by which the agent persists in nature from generation to generation of mink. Clearly, it will be some time before this disease is fully understood.

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Pathogenesis of Aleutian Disease of Mink¹

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Aleutian disease of mink is an important spontaneous disease which may help fill gaps in our knowledge of several human ailments. During its various stages, Aleutian disease (AD) possesses manifestations common to some of the human connective tissue diseases. Among these are marked plasmacytosis of the liver, spleen, and lymph nodes, extreme rise of serum globulin, smudging of glomerular basement membranes, and segmental periarteritis with fibrinoid degeneration (figs. 1 and 2).

Perhaps most intriguing of all is the fact that AD is caused by a filterable agent. There is considerable evidence that this agent is a virus. Pellets formed in the ultracentrifuge after 95,000 g for 1 hour are infective (1). It has been serially passaged 13 times through mink (2). It reaches titers of $10^{5.5}$ ID₅₀ per ml. of tissue suspension (3). It remains infective after fluorocarbon extraction (4). Final proof awaits critical experiments of localizing the site of virus proliferation in tissues and relating numbers of particles visualized in the electron microscope to biological activity.

It is in its context as a chronic viral disease that AD will be discussed here. In order to place this emphasis, we must make some assumptions which, as mentioned above, are only partly supported by experimental data. These are: (1) a virus initiates the disease; (2) this

leads by some mechanism to plasma cell proliferation and hypergammaglobulinemia; (3) there is resultant (or concomitant) damage to vital functions because of injury to liver, kidneys, and arteries.

Let us examine some of the possibilities related to these assumptions. The evidence we have accumulated concerning the etiology points toward some type of viral agent. We have not yet characterized it physically and must do this before more dogmatic statements can be made. The biological and biochemical data discussed by Burger et al. (4) are fairly convincing that some protein-covered, particulate, replicating entity is involved. The road to proof of a viral agent is a long and rocky one, however. Christian (5) recently reported the following concerning the etiology of rheumatoid arthritis (RA):

These observations have a sobering influence on anyone attracted to the thesis that a parasitic agent or agents are active in the pathogenesis of RA, and they suggest that demonstrating the microbes, if they exist, will not be easy. If a microbial agent responsible for human disease were as difficult to characterize as the agent involved in Aleutian disease of mink, present methods might not permit its detection.

By what mechanisms might such a disease syndrome be initiated and perpetuated by a virus? Until we have defined the intracellular site of attack, we can comfortably speculate without fear of immediate disproof. Four different possible mechanisms have been proposed by Leader (6). These involve (1) creation of a state of autoimmunity by alteration of self protein to form antigen; (2) alteration of protein followed by formation of protein-virus complex which then acts as antigen; (3) direct plasma cell stimulus to produce antibody; or (4) direct plasma cell stimulus to produce non-antibody globulin. Each of these hypotheses must deal with the chronic progressive nature of the

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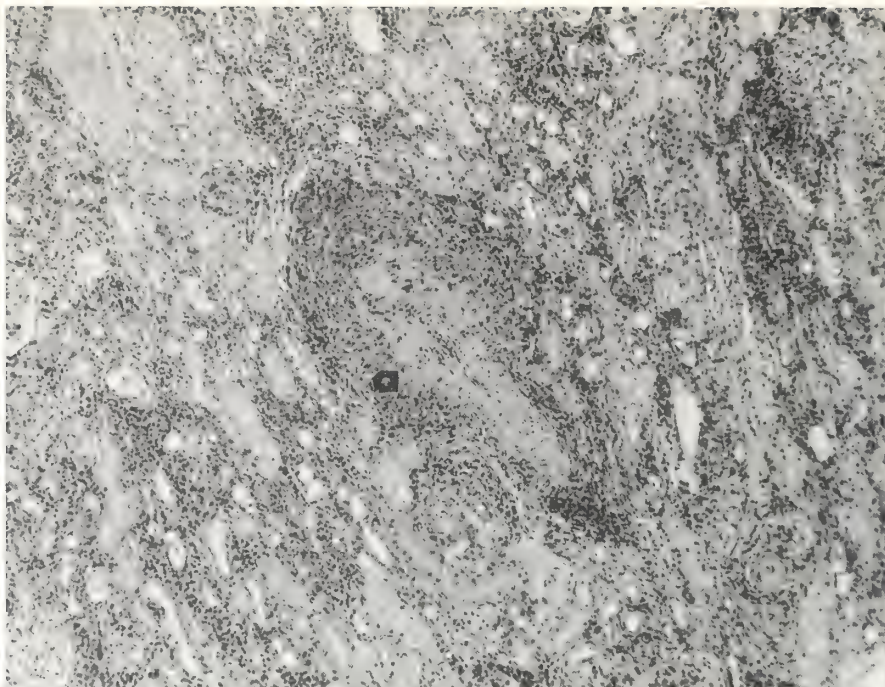


FIGURE 1.—Kidney of mink with Aleutian disease. There is heavy infiltration of cortex with plasma cells. (a) One artery shows periarthritis and fibrinoid degeneration of the wall. H. & E. $\times 100$.

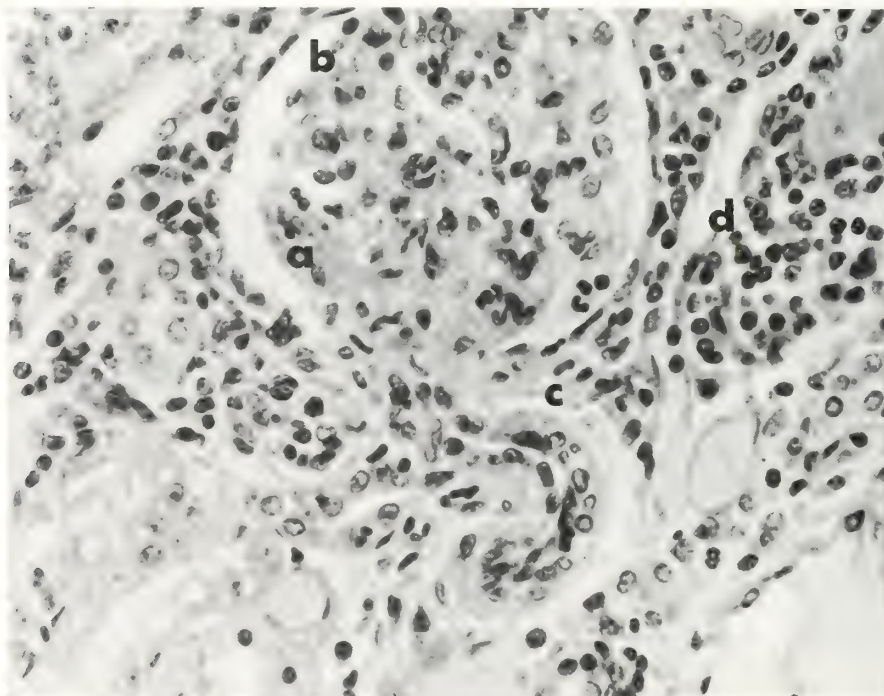


FIGURE 2.—Kidney of mink with Aleutian disease. (a) There is fibrinoid degeneration of the glomerular basement membrane, and (b) some evidence of pyknosis of nuclei with plasma cells. (a) One artery shows periarthritis and fibrinoid degeneration of interstitium can be seen. H. & E. $\times 375$.

disease, presence of virus proliferation throughout its course and existence of the active agent in high titer in the serum of animals with gamma globulin levels as high as 50 percent of total serum proteins (3). This is mostly 7S globulin as pointed out by Kenyon et al. (7), which would ordinarily be regarded as antibody. However, it is not functional antibody, at least by the measurements we have applied thus far (table I). It

TABLE I.—Neutralization Tests

Serum source	Virus ID ₅₀	Results infected/exposed
AD infected mink	>10 ⁴	12/14
Normal mink	>10 ⁴	15/15
Normal mink	None	0/5
AD infected mink	None	5/5

is not likely that we will know whether it is antibody until some in vitro method of measuring replication of virus is achieved. Basur et al. (8) have reported replication of the agent in tissue culture. Porter and Dixon (9) have reported that there were in addition to 7S globulin other components showing sedimentation patterns of 10S, 17S, 22S, and 25S. With a tissue culture system, such sophisticated technics as those used by Svebag (10) to study ontogeny of poliovirus antibody could be applied to measure type and rate of globulin production by plasma cells.

We can speculate that some unusual cell-virus mechanism must operate in a system where cellular proliferation, globulin production, and virus replication continue in concert. Much of the evidence accumulated up to now indicates that the plasma cells are stimulated to produce "junk" gamma globulin. If this is the case, it may be that the virus carries information into the host cell which diverts the activity of unprogrammed ribosomes toward production of viral RNA, while not interfering with the manufacture of DNA-dependent (normal cell) RNA. Hausen et al. (11), in comparing the intracellular effects of Maus-Elberfeld (ME) and fowl plague (FP) viruses, showed how ME virus drastically alters cell metabolism by cutting off synthesis of cellular RNA and protein. In FP infection, on the other hand, virus multiplication occurs as an additional performance of the cell. Hausen et al. (11) say, "Fowl plague virus lies in the periodic table between those viruses which lead to drastic breakdown of normal synthetic machinery and those apparently able to stimulate this machinery."

Franklin and Baltimore (12) believe that the cytotoxic viruses must kill cells to multiply, while tumor

viruses multiply but also allow the host cell to remain viable. In their studies of Mengo virus, they found that synthesis of DNA-dependent (normal cellular) RNA and protein was inhibited very early while DNA production dropped somewhat later. The inhibition of RNA and protein synthesis, they felt, was due to inhibition of normal RNA programming of host ribosomes. These unprogrammed ribosomes could then be programmed by viral RNA and diverted to synthesis of viral RNA and viral coat protein.

If we speculate how this idea might apply to AD, we could propose a modification of the scheme presented by Franklin and Baltimore (12) (fig. 3). We have no special justification for assigning AD virus to the group of RNA viruses except that the work with FP, ME, and Mengo viruses is concise to the point where their use as comparisons become convenient. Some system with DNA virus such as Shope papilloma as described by Ito and Evans (13) might also fulfill the types of mechanisms involved in AD. Shope (14) has speculated concerning similar processes which might operate in tumor viruses. In either case there could result a cell-virus system where the rate of virus synthesis was lower than in virulent viruses. Such a system might utilize only the unprogrammed ribosomes available, allowing synthesis of messenger RNA and protein to continue or even be stimulated. Chronic cellular proliferation, sustained hypergammaglobulinemia, and continued viremia are all consistent with this scheme.

An explanation for lack of antiviral antibody can be visualized if the target cell of AD were the plasma cell or reticular cell, which, in addition to being instructed to produce virus, were also caused to manufacture its ordinary secretion (gamma globulin) in slightly altered form.

This would place AD as a metabolic aberration between cytotoxic viruses, where synthetic mechanisms are all diverted to production of viral RNA and viral protein, and such tumor viruses as Rous sarcoma where the cells are transformed toward virus production but with no obvious impairment of their cellular RNA synthesis.

If this kind of mechanism were indeed in operation, and if all plasma cells were attacked in this manner, there should be some alteration in the ability of an affected host to produce antibodies against other stimuli.

It has been found by Gorham (15) that AD infected mink can produce antibodies to botulism toxoid and distemper virus. This question could be tested experimentally by first initiating AD infection followed by inoculation with other antigens after which successive

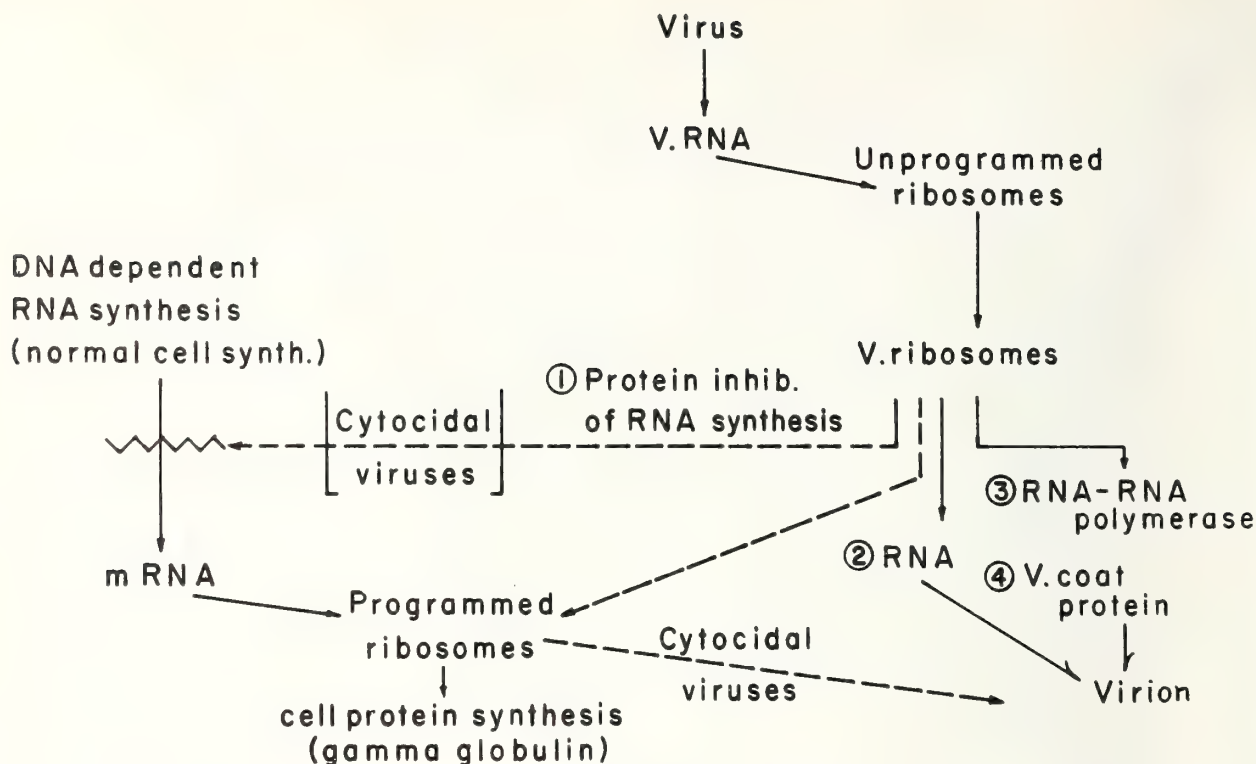


FIGURE 3

measurements of specific antibodies to botulinum toxoid, distemper virus and other antigens could be measured.

On the other hand, the virus might induce somatic mutation of certain plasma cells resulting in the subsequent multiplication of their progeny as forbidden clones. One could easily see resultant continuous secretion of abnormal gamma globulin from such transformed cells, but it would then be necessary to provide a separate mechanism to account for continued virus production.

If we support either of the mechanisms outlined above, we come close to categorizing AD as a low grade or slow neoplasm. It really becomes a semantic question whether one might consider an autoimmune disease with multiplication of forbidden clones of cells as a "controlled" neoplasm. Dameshek et al. (16), in a discussion of mechanisms of autoimmune diseases, allude to this possibility in lymphoproliferative diseases. Lymphoid tissue throughout the body becomes involved in AD as one can readily see when observing the evident plasmacytosis of liver, bone marrow, spleen, and lymph nodes. Examination of thymus glands from infected mink (17), however, failed to reveal the presence of germinal centers as reported by Holmes and Burnet (18) in NZB mice.

When we shift from considerations of etiology and metabolism in plasma cells to the actual mechanisms of tissue damage in AD at the organ level, we can still only speculate concerning the mechanisms involved. Since we really do not know where the agent attacks the body, it is not easy to pick a defensible anatomic locale for our pathologic dissection to begin. On the other hand, it is difficult to deny any of the several target organs; so in order to establish a reference point, let us start with the liver.

The inflammatory lesions and bile duct proliferation which develop in mink with AD (fig. 4) are most intriguing but of unclear relationship to the syndrome. These appear quite early, perhaps as soon as 2 weeks after virus inoculation and at about the same time that serum gamma globulin begins to rise. Experiments now being conducted by Leader et al. (19) show the bile duct proliferation, plasma cell accumulation and rises in serum transaminase occur almost simultaneously (fig. 5 and table II). Whether this represents localization of an antigen-antibody reaction is not clear. Popper (20) believes that portal inflammation in drug reactions may be hypersensitive. There is some resemblance of AD to plasma cell hepatitis of man, which also may be autoimmune in origin. Also in chronic ethionine intoxication there is infiltration

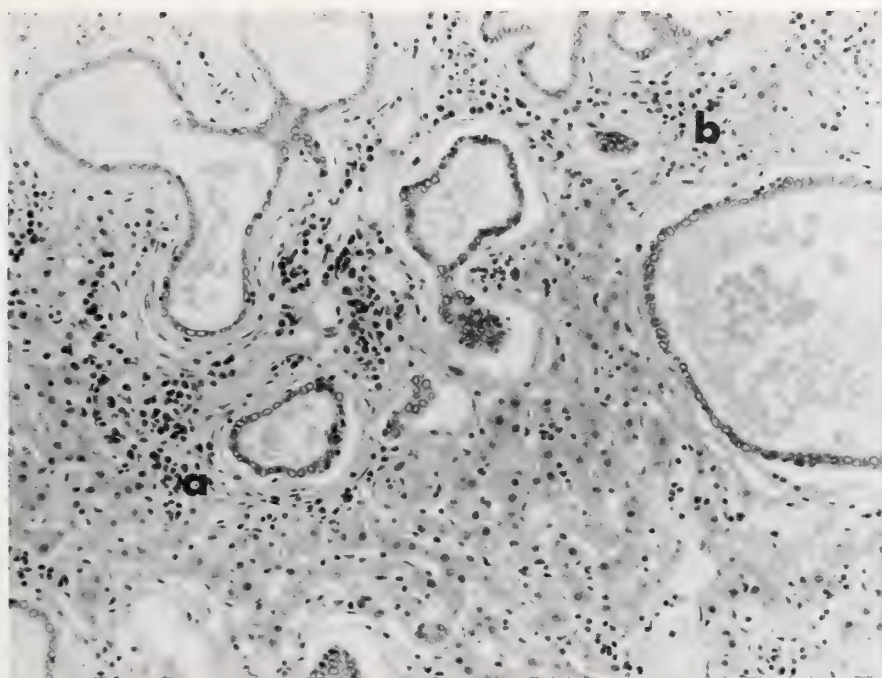


FIGURE 4.—Bile duct proliferation and dilatation. (a) There is plasma cell infiltration surrounding ducts; (b) hepatic necrosis and fibrosis are seen. H. & E. $\times 180$.

TABLE II.—Portal Plasmacytosis and Early Hypergammaglobulinemia

Days after challenge	Group			
	Control		Infected	
	Portal plasma-cytosis	IAT status	Portal plasma-cytosis	IAT status
1	—	—	—	—
3	—	—	—	—
5	—	—	—	—
7	—	—	1+	—
9	±	—	—	+
11	—	—	1+	—
15	—	—	±	—
18	—	—	3+	—
21	1+	—	3+	—
25	—	—	2+	+
30	—	—	4+	+
36	±	—	4+	+
43	—	—	—	+

of the portal triads with plasma cells. Popper cites these observations as support for his hypothesis that the liver can participate in autoimmune reactions. Complement fixing antibodies have been demonstrated by Gajdusek (21) but were not organ specific. Paronetto et al. (22) found that serum of many patients with liver disease contains antibodies against proliferated bile ductules and their contents, which did not

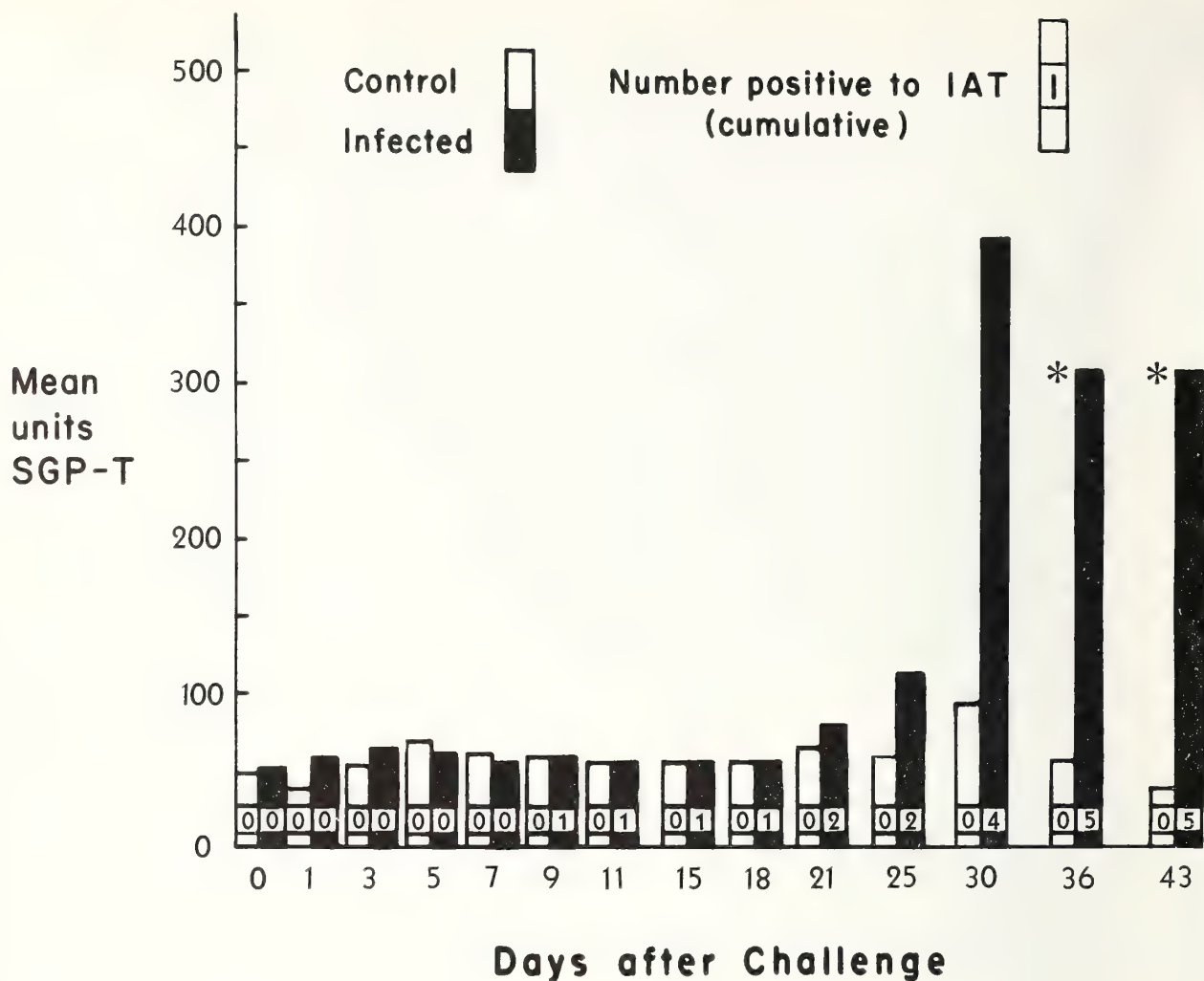
react with normal liver. The reaction was organ but not species specific; as human serum was also bound by proliferated bile ductules of rats intoxicated with thioacetamide.

Popper (20) presents the hypothesis that in some types of liver injury an irritant substance is released into the duct lumina by damaged tissues causing local inflammation and further bile duct proliferation. As a result local antibody formation may be stimulated leading to formation of antigen-antibody complexes which cause piecemeal necrosis of hepatic cells.

This reaction could then be perpetuated by the passage of the irritant through the bile ducts to other parts of the liver. Popper (20) reported that retrograde injection into rat bile ducts of antigen-antibody complexes from horse serum solubilized in excess antigen caused peribiliary inflammation which did not occur when antigen or antibody alone were given. He did not state what antigen was used.

Leader et al. (23) have described in some detail proliferation of bile ducts containing mucopolysaccharide to be seen in AD (fig. 4). Also they reported a rather unusual type of nonreactive necrosis of hepatic parenchyma. These lesions are similar to those described by Popper (20).

It is possible that the principal target in AD is the periductular tissue of the liver with subsequent lesions



* These levels include animals killed on days 30 and 36

FIGURE 5.—Graph illustrating reactivity of mink during the first few weeks after inoculation with Aleutian disease.

in other parts of the body resulting from this hyperergic reaction. Consideration of such a possibility brings to mind the experiments of Dixon (24) with serum sickness in rabbits. "One-shot" serum sickness, it must be remembered, results from injection of any of a large number of purified serum protein antigens, none of which need have any demonstrable antigenic relationship to the cardiovascular, renal or other tissues injured. These lesions closely resemble those of the acute stages of lupus erythematosus, rheumatoid arthritis, etc., but are short in duration as the animals recover rather quickly. Dixon (24) used a cleverly designed experiment to simulate the pathogenetic mechanisms operating in more chronic immunologic diseases.

He did this by injecting small amounts of purified protein daily into rabbits, in hope of creating a situation in which antigen-antibody complexes might be present over a long period of time. Individual animals reacted differently (1) by making copious antibody and quickly eliminating antigen; (2) by making no antibody allowing antigen to circulate; (3) by making an amount of antibody insufficient to remove antigen but capable of forming antigen-antibody complexes which persisted in the circulation until the next injection.

Animals producing excess antibody went through a phase similar to "one-shot" serum sickness at about 2 weeks, as they passed from antigen excess to antibody excess balance. Animals which produced no antibody

exhibited no abnormalities. Those animals which produced a small amount of antibody resulting in circulating antigen-antibody complexes developed chronic glomerulonephritis.

This work illustrates that tissues damaged by antigen-antibody complex need not be related immunologically to the antigen involved so that antigen-antibody complex formed by a reaction in the liver could conceivably cause tissue damage in kidneys, arteries, and other organs.

Some problems in this hypothesis as far as AD is concerned, however, are (1) that large amounts of circulating antigen-antibody complex have not been demonstrated, (2) that there appears to be marked antigen excess in the blood stream if the viral agent is the antigen, and (3) that attempts to localize antigen-antibody complex in damaged tissues have failed.

It could be that the antigen-antibody complexes formed are quickly deposited in tissues and therefore removed from the blood stream, but the observations of Henson (25) that fibrinoid degeneration of arteries occurred only when percentages of serum gamma globulin reached 50 percent of TSP make it seem more likely that the tissue damage is due to direct action of 7S gamma globulin rather than complexes. Porter and Larsen (26) also have reported a direct relationship between levels of gamma globulin and severity of lesions.

Porter and Dixon (9), in reporting the presence of high molecular weight globulin in mink infected with AD, stated that dissociation of the material in acid and urea buffers indicated that it was a protein-protein complex. Similar complexes are seen in some connective tissue diseases, but in glomerulonephritis and serum sickness induced as described above by Dixon the amounts were too small to detect with the ultracentrifuge. Campbell (27) pointed out that in extreme antigen excess there is a combination of two antigen molecules to one antibody (Ag_2Ab). This ratio of complex was not biologically active but Ag_3/Ab_2 and lower ratios were active and fixed complement. It may be that in AD the mixture of molecules of protein-protein complex is largely of Ag_2Ab but that small amounts of Ag_3Ab_2 or less exist, which could account for the slow progress of the disease.

We must then consider how such a hypothetical process might relate to lesions in the kidneys and arteries. The kidney lesions appear at a very early time, perhaps almost as soon as those in the liver. These consist of thickening of the mesangium of the glomeruli, cortical infiltration with plasma cells, hyaline tubular casts, and destruction of the proximal

convoluted tubules. If there is an autoimmune process initiated within the liver, the kidneys might very well become involved because of being subjected as the body sink-trap to antigen-antibody complexes passing through.

DISCUSSION AND SUMMARY

As we approach these complex biological phenomena, those of us who direct our interest toward comparative pathology must always feel drawn to ask the question, "What analogies to human disease can be drawn from our observations?"

When we look at diseases by systems or with etiologic considerations, we can easily lose the red thread and thrash frustratingly close to but unknowing of the answers. Only when we can establish common denominators in mechanism do we assault the truth. This is why we have left the safe pathways of straightforward pathologic anatomy and ventured into the world of the virologist and biochemist. There have been presented first a hypothetical mechanism to account for the progression of AD in time, after which there have been outlined some possibilities concerning how this activity might produce the lesions which subsequently develop. Now as we look back over these two divisions, it may appear to be difficult to fit them together.

If plasma cells, attacked by virus, were to produce first a protein which caused periportal irritation and damage to hepatic parenchyma the series of events could be set in motion by the resultant production of protein-protein complexes with ability to damage tissues. The plasma cell proliferation would be expected to continue concurrently as a direct result of viral stimulus.

Dameshek et al. (16) discuss three principal possibilities in development of autoimmune phenomena: (1) hapten mechanism where a chemical may attach to body protein creating an antigen; (2) release of previously sequestered antigen as in chronic thyroiditis; (3) graft versus host reaction resulting from alteration of body cells so that "forbidden clones" then grow, producing antibodies against self tissues.

Mechanism number three has great appeal when considered in light of the ideas of Franklin and Baltimore (12) but it seems difficult to reconcile this with the concept of autoimmune hepatitis. In their discussion of human plasma cell hepatitis, Page et al. (28) reported marked improvement of plasma cell hepatitis from 6-mercaptopurine in which liver functions

returned to normal even though hypergammaglobulinemia and plasma cell proliferation continued. They postulated a viral mechanism for the liver damage and suggested that 6-MP inhibited viral replication.

In order to address these hypotheses many experiments are available, including the following:

1. Study of ultrastructure of hepatic tissues and plasma cells. This is being done in our laboratory. From this we may localize the intracellular viral assembly points.

2. Examination of density gradients and pelleted material from ultra-centrifugation for particulate structures.

3. Production of fluorescein tagged antiserum against fluorocarbon extracted AD antigen followed by staining and examination of plasma cells, liver, kidneys, etc., to localize viral antigen.

4. Chemical breakdown of the viral material to determine its composition (29).

5. Study interspecies susceptibility. Some trials have been initiated where tissues from human cases of rheumatoid arthritis were inoculated into mink. The tests have thus far been negative. Kenyon and Desel (30) may have achieved transmission of a chronic human lymphadenopathy to mink with production of AD-like syndromes.

6. Administration of immunosuppressive agents followed by careful measurements of metabolic parameters such as serum proteins, SGOT, and SGPT. Studies of similar nature by Page et al. (28) in plasma cell hepatitis revealed that some functions returned to normal while others remained abnormal.

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DISCUSSION

FIELD: What happens if you challenge these animals with a strange antigen while they are developing the disease?

LEADER: This is one of the things that must be done. Dixon, I believe, did some experiments in which he challenged his mink with foreign proteins and found that the rise in gamma globulin characterized by the 7-S material in Aleutian disease was independent of the response against other antigens.

EDDY: I was wondering if you have tried any tissue cultures of the mink kidney or any other mink tissues?

LEADER: There has been some work going on in our laboratory in a small way. Dr. Salk has a task force of considerable size turned loose on this. Basrur, Gray and Karstad in Canada have reported proliferation of the virus in tissue culture. I agree that one of the really important things to do is to get an in vitro system established.

BROTHERSTON: How about transmission to other animals?

LEADER: It has been tried by us and by others without success. I have tried it in cats. Cats have some interesting properties in common with mink, but so far we have had no luck. Nor have we been successful with ferrets. Other people have tried it in ferrets and mice.

HADLOW: We have tried it in mice without success.

BANG: Did you make dilutions of your serum? Is it possible that your failure to neutralize is a prozone phenomenon?

LEADER: It is very possible, and one of the things that we have been planning to do is to separate the virus that is present in the serum from the gamma globulin and then do these titrations again. One of the problems here, of course, is that the mink are born during one period of the year and all titrations must be performed in mink. We have 500 or 600 animals under experiment every summer and the number of experiments which can be performed is limited.

BROTHERSTON: Does the experimental disease go in all genotypes?

LEADER: Yes, in all genotypes. However, there is greater susceptibility in homozygous recessives compared to other genotypes.

FIELD: Is there anything in the thymus in these animals?

LEADER: The thymus glands that we have looked at have been normal. Dr. Burnet was in our laboratory this spring and looked at a number of thymus glands. He could not find any germinal centers in them.

HOTCHIN: How far can you press the analogy with multiple myelomatosis?

LEADER: I think it would be stretching a long way to call it a multiple myeloma and I certainly do not; but I think if we establish that virus attaches to reticular cells directly or to the plasmablast directly and causes them to proliferate in large numbers and produce an ineffectual nonantibody type of gamma globulin, then we are approaching the zone of neoplasia.

Transmissible Encephalopathy of Mink¹

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Encephalopathy of mink has been recognized on a few Wisconsin mink farms during the past 15 years. Further observations gathered during a severe epizootic in the summer of 1963 have left little doubt that this disease is distinct from any known neurological disorder of mink (1, 2). While many factors regarding etiology and pathogenesis of this disease remain to be determined, its clinical and pathological features are reminiscent of sheep scrapie. At about the same time, Hadlow (3) observed a similar condition in Idaho.

This paper describes certain clinical and pathological features of what we will provisionally call mink-encephalopathy. In addition, some findings regarding epizootiology and etiology of the disease are presented.

Clinical Signs

The clinical signs are characterized by progressive central nervous disturbances and locomotor incoordination. The manifestations develop slowly, almost imperceptibly. The first signs are subtle changes in the normal habits of feeding and cleanliness. Some animals become more active and excitable when approached. Occasionally, convulsions are recorded. The tails of the animals are carried abnormally high, arching over the body. Hearing and vision appear to remain unimpaired and corneal and pin-prick reflexes are present. Often times, the animal runs or walks in circles and exhibits involuntary foot movements. Animals with severely mutilated tails are frequently seen.

Somnolence of progressive severity is a consistent feature (fig. 1).

Soon the gait of the mink becomes stiff and its footing uncertain. A typical jerky stepping action of the hind legs is observed. Later, the locomotor disturbances progress until the animal is almost completely incapacitated and unable to move. There is no evidence of flaccid paralysis during the course of the disease. Terminally, loss of bodily condition becomes more and more apparent. The animal may sit in a corner of the pen with its teeth attached to the surrounding wire mesh. A fine tremor is often present. After a course, which may last from 3 to 8 weeks, death ensues. The case fatality rate is 100 percent.



FIGURE 1.—Mink with advanced encephalopathy. The animal bit a protective mitten while being handled. Then the leather mitten was removed and placed with the animal on the ground. The mink remained almost motionless for about 15 minutes in the position shown, an observation which is never made with healthy mink. Reproduced with permission from the *Journal of Infectious Diseases*, 115:4 (October), 393-399, The University of Chicago Press. Copyright 1965 by The University of Chicago.

¹ This study was performed at the Department of Veterinary Science, University of Wisconsin, Madison, Wis. and the Diagnostic Laboratory, Great Lakes Mink Association, Pittsville, Wis.

Pathology

The carcass is usually dehydrated with greatly reduced adiposal deposits. No consistent macroscopic or microscopic abnormalities are present in the striated muscles, bones, and visceral organs of the affected animals. An occasional animal may exhibit a slight edema of the brain.

Microscopic lesions in the brain are widespread and invariably present. Most impressive is a diffuse vacuolation of the grey matter, giving this area a spongy appearance (fig. 2). Prominence of astrocytes is a consistent part of the disease (fig. 3). Various forms of neuronal degeneration such as chromatolysis, sclerosis, and pyknosis are seen in many areas of the cerebrum, the brainstem and the cerebellum. In the dorso-ventral section through the cerebellar peduncles some neurons are found which contain cytoplasmic vacuoles (fig. 4). These vacuoles may be small or so large as to replace the cytoplasm almost in its entirety.

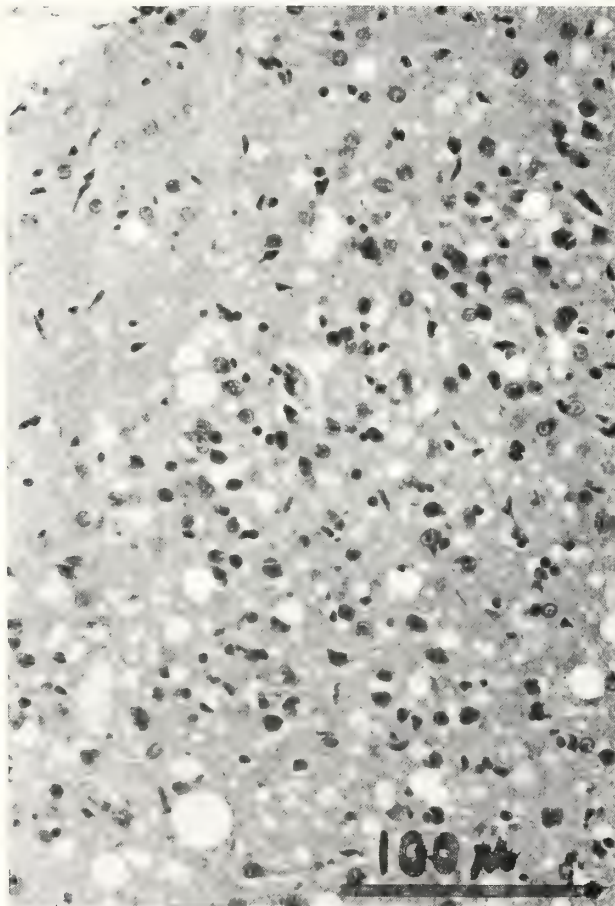


FIGURE 2.—Natural case of mink encephalopathy. Vacuolation of the grey matter, increased cellularity and neuronal degeneration in the rostral part of the cerebral hemisphere. (H. & E.)

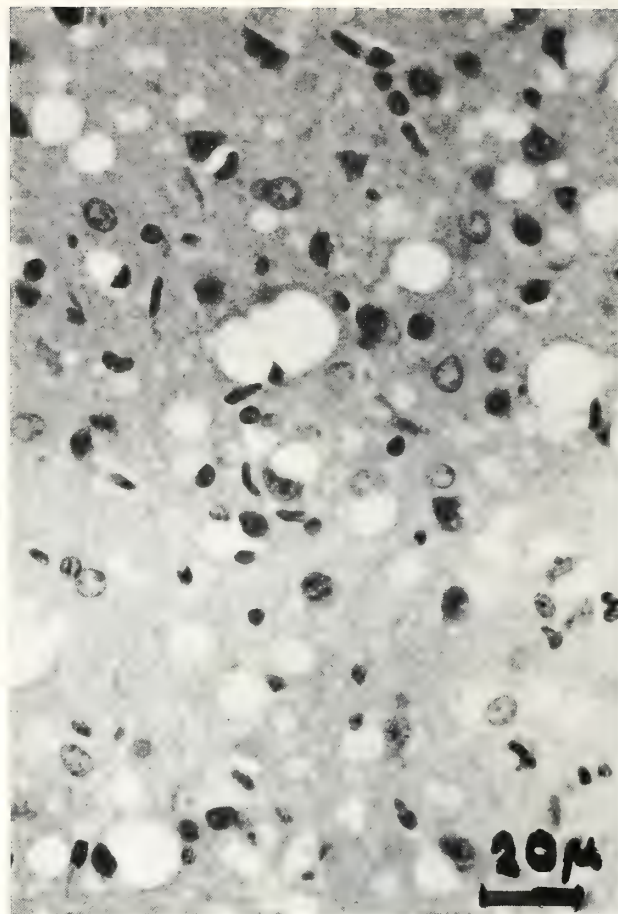


FIGURE 3.—Natural case of mink encephalopathy. Proliferation of astrocytes in the grey matter. (H. & E.)

Often they appear “empty” or contain a few small granules which stain readily with eosin. In our limited study neuronal vacuolation was most impressive in the dorsal and medial nuclei of the cerebellar peduncle.

In summary, the characteristic lesions of mink encephalopathy are widespread neuronal degeneration with astrocytosis and spongy degeneration of the grey matter. A localized prominence of vacuolated neurons is present. A general resemblance of these changes to those described as characteristic for scrapie (4, 5) is apparent, although differences regarding their topography cannot be denied.

Epizootiology

Mink differ in many respects from other farm animals and a brief description of their biology and husbandry as pertaining to this discussion is given.

As with wild mink, ranch mink have only one breeding season a year, mating during March. Four to seven mink kits per litter are born after a gestation

period which averages about 50 days. The dams nurse their young for about 4 to 6 weeks; however, during the third week of life they will begin to eat some solid food if it is provided.

Mink kits have a remarkable growth rate, 80 percent of the final body weight being reached within 5 months, by September of the same year. The development of the winter pelt is noticed at this time. After the mink are prime, usually in November or December, most of these kits are pelted. Only those animals which have been selected for the new breeder herd are kept through the winter. The reproductive cycle is then repeated. Commercial considerations require a certain size for the breeder herd, ranging from about 400 to 5,000 animals or more. Usually one male is provided for 4 females. During most of their lives mink are confined individually to wire mesh pens

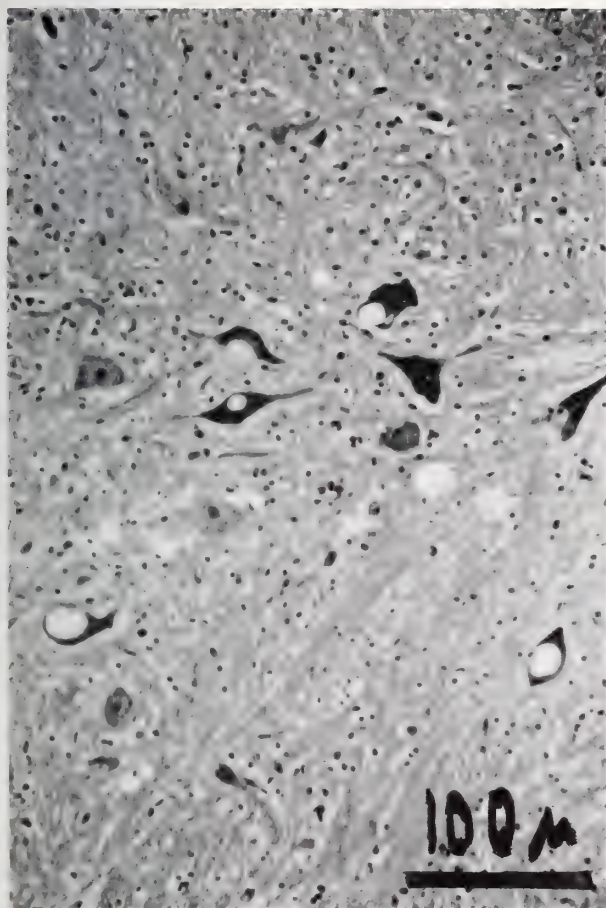


FIGURE 4.—Neuronal vacuolation in the cerebellar preduncles. (H. & E.) Reproduced with permission from the *Journal of Infectious Diseases*, 115:4 (October), 393–399, The University of Chicago Press. Copyright 1965 by The University of Chicago.

which are assembled in rows and located either in the open or in sheds.

In the wild, the mink is a solitary killer with a discriminating taste for fresh fish, frogs, small mammals and birds and insects. The diet of today's ranch mink, although modified by nutritional research, traces its origin to the food consumed by mink in the wild state. The ingredients of this diet may vary from season to season or ranch to ranch depending on the nutritional requirements or differences in availability and price of the raw materials. Most rations are based mainly on fish, raw meat, and packinghouse and poultry by-products, to which cereal products supplemented by vitamins, trace minerals, antibiotics, and other drugs or chemicals are added.

As most of the ration ingredients must be processed without the benefit of heat sterilization, feed may be a source for not only nourishment but also for disease and parasites of mink. Botulism, anthrax or tularemia are not uncommon following the feeding of contaminated feed.

At this writing three separate epizootics of mink encephalopathy involving a total of seven or eight Wisconsin farms have been observed. The first epizootic was noted in 1947. A few comparatively mild outbreaks occurred in 1961 and a more severe epizootic was seen in 1963. The condition affected only adults of both sexes.

a. 1947.—The first experience of one of us (G. R. H.) with the disease was on Farm A in Brown County, Wis., in the fall of 1947 (fig. 5). In late November a few adult mink of the breeder herd developed locomotor incoordination which was followed by somnolence and progressive debilitation and terminated in death. During the following 4 months the losses increased, accumulating finally in a total of about 1,200 mink.

At the onset of this outbreak the breeder herd consisted of 5,250 mink, 4,000 of them were born in May 1947, the remaining 1,250 animals were from previous whelping seasons. The disease appeared only in mink over 1 year of age, even though the younger animals ate the same feed as the older mink and were housed together with the dam from May until September. Then all mink were placed in individual pens. Among the older mink the morbidity and consequent mortality reached 99 to 100 percent, in contrast to no deaths attributable to the condition in the younger group.

The mink were fed the usual diet of raw meat, packing plant by-products, fish, liver, and cereal. On Farm A, meat from dead or paralyzed cattle ("downer"

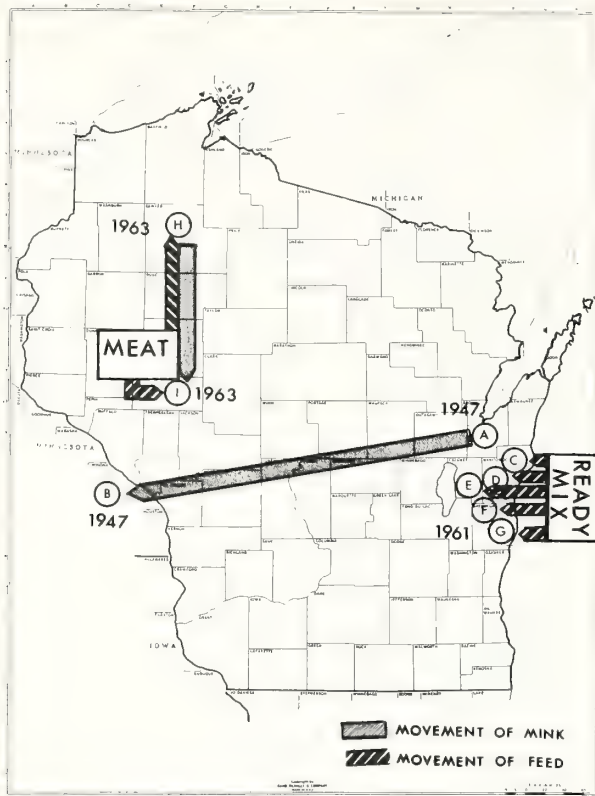


FIGURE 5.—Encephalopathy of mink in Wisconsin in 1947–63. For explanation see text. (Map Copyright by Rand McNally 65W21). Reproduced with permission also from the *Journal of Infectious Diseases*, 115:4 (October), 387–392, The University of Chicago Press. Copyright 1965 by the University of Chicago.

cows) was used as part of the meat ration. Two injections of a formalized homologous tissue vaccine were given for distemper prevention in June 1946, followed by a single booster injection of the same vaccine in February 1947.

Of particular significance was the fate of 125 mink which in April 1947 were shipped as bred females to Farm B in Winona County, Minn. (fig. 5). They whelped in May 1947 and raised their litters without difficulties. However, in late November the disease developed simultaneously on the ranch of origin and in the adult breeder animals shipped to Farm B. Other adult mink present on Farm B remained free of the disease. There had been no feed items in common and no contact with Farm A after April 1947. The case fatality rate here as on the ranch of origin reached 100 percent within comparable periods of time. None of the kits born in May 1947 were affected on either Farm A or B.

It was assumed that an unidentified toxic or infectious principle which must have been present on Farm A prior to April 1947 had caused the disease. The homologous tissue vaccine or feed consumed prior to April 1947 appeared as the most probable source. Since neither samples of the vaccine nor the diet fed at that time had been preserved, the investigation was discontinued. It was noted, however, that an unusually long period of time (6 months or more) must have elapsed between exposure and appearance of the disease.

b. 1961.—Beginning in July 1961 simultaneous occurrence of a similar disorder of the central nervous system was observed on several mink farms (Farms C–G, fig. 5) in Sheboygan, Calumet, and Manitowish Counties of Wisconsin.

Clinical signs and the course of the disease were identical to the previously described outbreaks on Farms A and B. Again only adult mink were affected. Kits nursing on dams that died 1 month later with the disease remained unaffected. In contrast to the 1947 epizootic, the incidence was less, varying among farms from 10 to 30 percent of the adults. All the farms involved were feeding a ready-mix feed ration which was prepared in a common feed plant. The use of the ready-mix feed was discontinued on January 1, 1961, by one of the farms. This farmer had prepared his own feed ration during the 7 months preceding the outbreak. The change of diet had no effect on the incidence or the outcome of the disease on his farm. It was assumed, at that time, that feed from the common feed source may have caused the disease. Again, a long incubation period, 7 months or more, must have passed between exposure to the toxic feed and appearance of the disease.

c. 1963.—During the summer of 1963 another epizootic was observed in Sawyer County, Wis. During the second week in May, locomotor incoordination was seen in some of the breeder mink of Farm H (fig. 5). In May the herd consisted of 1,178 adult mink and 4,500 newborn kits. The first deaths of affected mink occurred during the second week in June. At this time the dams were still with their litters. In the following weeks an increasing number of adults developed central nervous disorders. During July and August deaths from encephalopathy averaged 12 to 15 adults a day. The losses continued although at a lower rate through September and October and of the 1,178 adults only one animal survived. The one survivor was a mutation mink of unknown origin which was trapped in the wild and incorporated into the herd in April 1963. We feel that the survival of this mink is

of particular significance in that it may have been introduced into the herd after the rest of the herd had been exposed.

The destructive course of the disease in the breeder herd was contrasted by the total absence of neurologic disorders in the 4,500 kits, although these kits were housed under similar conditions and consumed the same feed and water as the affected adults. Kits nursed by females with early or moderately advanced disease remained healthy. In some instances the dam died while still together with her young.

In May 1963 the ration fed on Farm H consisted mainly of horsemeat, packinghouse and poultry by-products, fish, liver, and cereal. Earlier in March and April 1963 the diet was supplemented by carcasses of trapped wild beaver. During July through September 1962, meat from dead or "downer" cattle purchased from another mink farm, Farm I (fig. 5) in Eau Claire County, Wis. was part of the ration. It appears likely that meat from the same source and probably the same livestock carcasses was fed on Farm I at the time of butchering. The possibility of sheep meat being part of the diet cannot be excluded.

While investigating the outbreak on Farm H we became interested in the fate of 10 male mink born in May 1962. These mink were shipped on December 9, 1962 to Farm I. In June 1963 they developed signs of encephalopathy. While visiting Farm I, it was found that not only these 10 mink from Farm H, but also a number of other adults born on Farm I had contracted the disease. All affected mink died after a progressive course of the disease. By fall 1963 the mortalities on Farm I had exceeded 70 percent of the adults, with none of the kits born in 1963 being affected. All surviving mink were killed and pelted in late November 1963.

In the absence of other evidence we believe that either the movement of mink from Farm H to Farm I or the shipment of meat from Farm I to Farm H must have played a causal role in these outbreaks. It is quite obvious however, that any speculation regarding the etiology of these two outbreaks must reckon with not only the coincidental appearance of the disease on two farms separated by a distance of more than 100 miles, but also the apparent absence of neurologic disorders in mink kits which were present in large numbers on both ranches during a time of prevalence of the disease. After the 1963 epizootic we arrived at the following conclusions which admittedly were speculative:

(1) The disease did not spread readily from affected to nonaffected mink because the disease was self-limiting.

(2) The illness was observed in adult mink on both farms because these were the only ones exposed.

(3) A common source of exposure existed for both farms, which may have been the meat from dead or diseased livestock which was fed during the summer of 1962.

(4) The lengthy incubation period may have been the characteristic property of a causal agent present in the suspect feed.

Etiology

Repeated attempts to isolate bacterial pathogens from affected mink have, in our hands, failed to yield conclusive results. Common contaminants such as *Escherichia coli*, *Staphylococcus albus*, and unclassified fungi grew on the culture media employed but were considered accidental findings. The possibility that *Toxoplasma gondii* might have been the causal agent was considered, but rejected on grounds of absence of typical pathologic lesions and failure to isolate the agent by mouse inoculation. Several attempts to cultivate the suspected agent in bovine, porcine, avian and mink kidney tissue cultures, chicken embryos, and suckling white mice were also unsuccessful.

On the other hand, the disease could be transmitted to other mink with brain material. A crude 10 percent tissue suspension of mink brain in penicillin-streptomycin containing saline was used for these experiments. On a filtration experiment the suspension was clarified by centrifugation at 500 g for 30 minutes prior to filtration through a Seitz filter of 500 m μ pore size. Results of these experiments are presented in table I.

The mink employed in the transmission experiments were from 2 different sources; 10 of these animals were mink kits which had been on Farm H during the 1963 outbreak. Nine other mink were obtained from a farm which had never experienced encephalopathy.

These 19 mink were divided among 7 experimental groups (table I) so that each group comprised animals from both farms. The experiment was conducted on Farm H. The 829 mink of the breeder herd served as environmental controls. All mink received the same feed and were housed and cared for in a similar manner.

The mink were infected on September 25, 1963, or with the filtrate on October 7, 1963. During the first week of March 1964, some mink developed early signs of the disease. During the last week of March 1964, locomotor incoordination was seen in all mink previously injected with brain material. On March 26,

TABLE I.—Encephalopathy of Mink, Summary of Transmission Experiments

Inoculum	Number mink	Clinical encephalopathy
Unfiltered brain homogenate, 1 ml. i.m.	3	¹ 3/3
Filtered brain homogenate, 1 ml. i.m.	3	3/3
Ether-treated brain homogenate, 1 ml. i.m.	2	2/2
Formalin treated brain homogenate, 1 ml. i.m.	3	3/3
Boiled brain homogenate (15 min. 95C) 1 ml. i.m.	3	3/3
Unfiltered brain homogenate, 1 ml. fed	3	3/3
None (contact controls)	2	² 0/2
None (environmental controls)	829	² 11/829

¹ Numerator: Number of mink with encephalopathy.
Denominator: Number of mink exposed.

² For explanation see text.

one mink which had been inoculated with unfiltered brain material died from the syndrome. By the second week of April, a total of seven inoculated mink had died. The remaining four were killed in advanced stages of nervous disorder. The survival time (experimental injection to natural death) ranged from 184 to 197 days. The three mink which had ingested the brain material, developed the typical syndrome during the second and third week of May 1964. After a progressive course of the disease, they were killed early in June 1964. The contact controls did not develop the syndrome.

It is evident that an incubation period of about 8 months following alimentary infection of experimental mink is in good agreement with the epizootiological observations of the natural disease. After subcutaneous injection a shortened incubation period (5 months) was noted.

In the control herd a few mink were discovered with moderately advanced locomotor disorders in late February 1964. The signs were similar to those seen during the epizootic in summer, 1963. During March 1964, more cases developed, eventually accumulating a total of 11 mink. The scattered distribution of these new cases among the herd made mechanical cross-transmission an unlikely explanation. This notion finds additional support in our failure to transmit the disease experimentally by contact. When searching for other sources of infection, we found that five of these mink were from litters of which the dam had died

with encephalopathy during June 1963. These dams were eviscerated. Parts of their entrails and flesh were apparently ingested by their young. We assume that at least five of these kits had acquired the disease by ingestion of diseased flesh from their dams. For the remaining six cases, no conclusive history of possible exposure could be constructed.

DISCUSSION

Encephalopathy of mink has many characteristics of a slowly progressing virus infection (6). Experience with the natural and experimentally induced disease strongly points toward a lengthy latent period of more than 7 months after alimentary infection. Since the incubation period exceeds the average life expectancy of the pelted herd, the syndrome is usually recognized only in those animals set aside for breeding purposes. Under natural conditions the disease is apparently self-limiting. Only in the 1963 outbreak did we observe a few secondary cases which appeared about 9 months after the first epizootic had begun. The etiology of these cases can perhaps be explained best by cannibalistic ingestion of diseased flesh or the assumption of other unusual means to acquire the disease. Two of the epizootics were characterized by circumstantial evidence incriminating a food-borne agent which most likely was present in meat taken from dead or diseased livestock. Although the particulate nature of this agent or its unlimited power of replication remains to be established, experimental transmission and other evidence (filterability and ether-stability) favor the theory of a viral etiology. Some unusual characteristics must be attributed to this agent: it resists boiling for at least 15 minutes and survives inactivation by overnight incubation with 0.3 percent formalin.

In view of the long incubation period and the pathologic characteristics of this disease, it is noteworthy that an unusually great resistance towards heat and formalin is typical for the causal agent of scrapie (7). However, in the absence of practical serologic or immunologic tests for the scrapie agent, any further comparison will have to await the outcome of cross transmission experiments using sheep and mink.

SUMMARY

A new disease of mink characterized by progressive debility and locomotor incoordinations has occurred on a number of Wisconsin mink farms during the past few years. Epizootologic and experimental evidence points to an infectious agent of long incubation.

Resistance of this agent to boiling and treatment with formalin along with histopathology, which reveals largely degenerative changes in the brain, suggests a disease strikingly similar to scrapie or rida of sheep.

ACKNOWLEDGMENT

This study was supported in part by U.S. Public Health Service Training Grant 5 TIAI 1750 4 and a special grant from the Wisconsin Department of Agriculture 93-4454 (The Study of Long Incubation Diseases of Mink).

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DISCUSSION

HADLOW: I happened to have encountered the same disease in Eastern Idaho, in the same year as Dr. Burger's outbreak, though onset of the disease occurred on about the first of the year. I was fortunate enough in seeing both of these outbreaks. They are the same disease. In this particular instance, I know considerably less about the epidemiologic factors that may have a bearing on its appearance in this herd, but the same disease appeared, and, again, it affected the adults—though this was before the breeding season, so there were very few kits available. I have been impressed by the changes in the cerebral cortex, which are most pronounced in the more rostral parts of the cerebrum (fig. 1). The most striking feature is this spongy alteration of the gray matter. I have not been too impressed with vacuoles in nerve cells, probably because I have not looked closely enough for them. I have been impressed, as Dr. Burger has, with the astrocytic response (figs. 2, 3, 4). Many areas, such

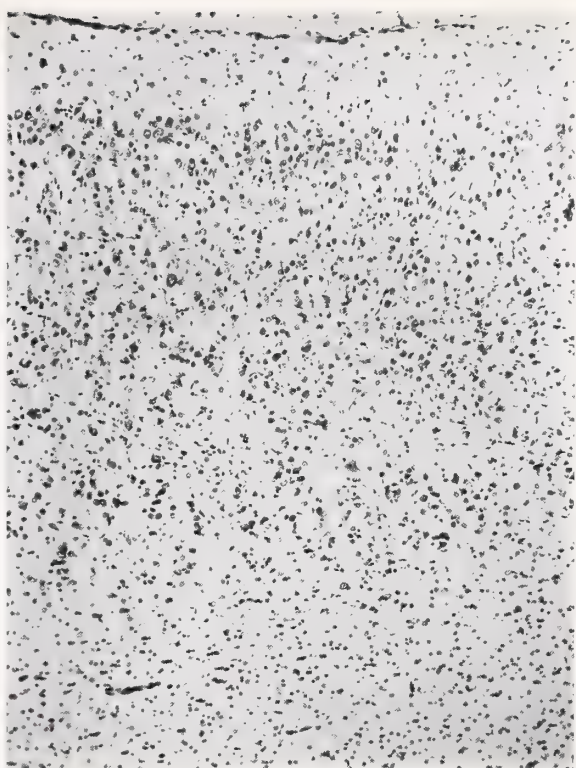


FIGURE 1.—Typical appearance of cerebral cortex characterized by shrunken neurons, increased number of astrocyte nuclei, and vacuolated parenchyma. Idaho mink killed in advance stage of the disease. Azure-eosinate. $\times 64$

as the thalamus and the caudate nucleus (fig. 3) show both the spongy appearance and the hypertrophy of astrocytes. In mink, astrocytes stain more readily than they do in mice, but in the normal animal stainable astrocytes are certainly less numerous and much more delicate in appearance than those in affected mink.

Arguing from the same basis that Dr. Burger has, we thought it might be interesting to see if this was, indeed, a transmissible disease. Accordingly, 12 mink were inoculated in 4 groups of 3. It so happened that three color phases, pastel, sapphire, and pearl, were represented in each group. We used brain and spleen from one of the naturally infected animals from the Idaho outbreak. This was made up as a 10-percent suspension and the animals were inoculated intraperitoneally with 0.5 ml. One group of three was inoculated with the unfiltered 10^{-1} suspension of brain; another group was inoculated with a filtrate that had been prepared by passing this preparation through a 450 $m\mu$ millipore membrane. This was done with the spleen also. After about 6½ months, the first animal became affected. This was one



FIGURE 2.—Astrocytosis in frontal cortex. Idaho mink that died of the disease. Cajal's gold sublimate. $\times 156$



FIGURE 4.—Intense astrocytosis in olfactory tubercle. Wisconsin mink killed in terminal stage of the disease. Cajal's gold sublimate. $\times 156$



FIGURE 3.—Enlarged astrocytes and spongiform change in caudate nucleus. Same mink as in figure 1. Cajal's gold sublimate. $\times 156$

that had received unfiltered brain. In due course, all of those that had received unfiltered brain became affected with the disease; somewhat later, in a matter of weeks, those inoculated with the filtered brain became affected, and then those that had received the unfiltered spleen. Finally, approaching a year after inoculation, the disease appeared in two of the three that had received filtered spleen.

Thus, this confirms what Dr. Burger found with material obtained from the Wisconsin outbreak, that the disease is transmissible and is induced after a long incubation period. We are now trying to get more information on the size of the virus and some idea of its distribution in the infected animal.

ZLOTNIK: If we compare this disease with scrapie—and the lesions certainly look identical—then it does not look like first transmission from a sheep or a goat to another animal. It looks as if it is already a passage. Is there any possibility of this sort of disease, this scrapie-like disease, existing in rodents, such as rats or shrews, with which the mink may be in contact?

HADLOW: Yes, there is. At present I have a completely open mind about how these animals became exposed.

BURGER: We must also consider the economical life span of these animals. They are held for profit and killed artificially and the economical life span of mink is only about 5 to 6 months. We have here a disease which has a longer incubation period than the overwhelming majority of that species' economical life span, so that we can see it only in breeding animals. This is a very important epizootiological fact.

ABINANTI: If one accepts this as scrapie in mink, then it brings up a lot of important epidemiologic ques-

tions. It would appear that these mink were fed beef, and it is conceivable that the disease is caused by a virus which is commonly present in cattle. This possibility of a silent host may also help to explain the varied epidemiologic patterns which are found in scrapie; in sheep, the silent host may actually be cattle. In a sense this also brings us back to the other very interesting question of kuru and cannibalism.

Some Physical and Chemical Characteristics of Partially Purified Aleutian Disease Virus

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Most workers feel that Aleutian disease (AD) of mink is caused by a virus. This hypothesis is based on the following: (1) serial transfer beyond the theoretical dilution end point (1); (2) transfer by cell-free tissue suspensions (2-5); (3) occurrence of AD in mink of heterogeneous genetic background (6); (4) the agent appears to be particulate as determined by filtration and sedimentation in the gravity field of an ultracentrifuge (7).

Filtered crude tissue preparations readily evoke the disease. However, the possibility of concomitant impurities influencing the response of the mink, particularly in relation to hypergammaglobulinemia, was not known. The development of purification procedures for this agent, and the general availability of such virus preparations would be one of the important steps toward greater understanding of this disease.

The absence of specific serological tests also favors a search for other methods of characterization of the agent. This report deals with preliminary efforts aimed at development of simple purification procedures and characterization of the agent by its resistance to chemical and enzymatic inactivation.

MATERIAL AND METHODS

Virus

The source virus for this study, the Pullman isolate, has been propagated serially in mink during the last three years. The passage history (table I) reflects some of the many experimental approaches which have

TABLE I.—Serial Passage History of the Pullman Isolate of Aleutian Disease Virus¹

Passage	Inoculum and quantity
<i>1961</i>	
1	Inadequately formalinized spleen suspension prepared as a vaccine.
2	Spleen, 1 ml. of 10 ⁻¹ suspension.
3	Spleen, 1 ml. of 10 ⁻¹ suspension (mink received dexamethasone after inoculation).
<i>1962</i>	
4	Resuspended pellet following centrifugation at 95,000 ref for 1 hour.
5	Untreated urine, 1 ml. undiluted.
6	Spleen, 1 ml. of 10 ⁻⁵ suspension.
7	Spleen, 1 ml. of 10 ⁻¹ suspension.
8	Whole blood, 1 ml.
<i>1963</i>	
9	Whole chicken embryo with yolk sac, 1 ml. of a 10 ⁻¹ suspension (3rd passage).
10	Spleen, 1 ml. of 10 ⁻¹ suspension.
11	Spleen, liver, kidney suspension, extracted 2 times with trifluorotrichloroethane, 1 ml. of 10 ⁻² suspension.
12	Serum, 1 ml. of 10 ⁻² dilution.

¹ From Gorham, J. R., Leader, R. W., and Henson, J. B. (7).

been used in this laboratory. The isolate is presently at the 13th passage level. It has retained its infectivity at -60° C. over a storage period of more than 2 years.

Ten percent tissue suspensions (weight/volume) in 0.1 M phosphate buffered saline were prepared by homogenization in a Waring blender. The crude suspension was subsequently clarified by centrifugation for 30 minutes at 3,000 g. The supernatant of

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this crude tissue suspension was employed in these experiments.

Mink

Two hundred and ninety-three mink of both sexes, 2 to 4 months of age were used. While most experimental animals were homozygous or heterozygous for the Aleutian gene, other color phase mutations were also represented. The mink were housed individually or in pairs within treatment groups and were fed the usual ration. All mink were negative when tested with the iodine agglutination test (IAT) (8) one day prior to inoculation. Each mink was injected intramuscularly with 1 ml. of the test suspension.

Criteria of Infection

Diagnosis of AD was based on: (1) the observation of positive iodine agglutination tests of increasing strength—every animal was tested 4 to 6 times during the 8 week duration of the experiment; (2) clinical signs; (3) presence of typical pathologic changes in kidney, spleen, and liver at autopsy; (4) presence or absence of microscopic lesions in animals which reacted doubtfully to the iodine agglutination test.

Protein Determinations

Protein nitrogen was determined by the Kjeldahl technique.

RESULTS

Heat Precipitation of Concomitant Proteins

Our first attempt to remove impurities from tissue suspensions was by heat precipitation. Ten percent tissue suspensions prepared from liver, spleen, and kidneys of affected mink were adjusted to various hydrogen ion concentrations as indicated in table II using 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. All suspensions were heated for 15 minutes at 60° C. After rapid cooling the suspensions were clarified by centrifugation for 30 minutes at 5,000 g. The pH was then readjusted to neutrality. The infectivity assays and protein concentrations of these suspensions are shown in table II.

While this experiment revealed a marked heat stability of the agent at a wide range of pH values, the degree of agent purification obtained was not satisfactory. The protein content of the test suspension heated at pH 3.4 was reduced only to 60 percent of the unheated control (table II).

Fluorocarbon Extraction

The use of fluorocarbons for removing host cell components from aqueous suspensions leaving a relatively pure virus suspension was first suggested by Gessler et

TABLE II.—Heat Resistance (60° C. for 15 Minutes) at Various Hydrogen-Ion Concentrations

Dilution	pH 3.4	pH 4.3	pH 5.2	pH 6.2	pH 7.2	pH 8.0	pH 9.0	Unheated control pH 6.8
	Protein Percent Original							
	63	77	85	89	98	95	90	100
10 ⁻¹	14/4	4/4						
10 ⁻²	3/4	4/4	4/4			3/4	3/4	
10 ⁻³	3/4	4/4	4/4	4/4	4/4	3/4	3/4	4/4
10 ⁻⁴			4/4	4/4	3/4	2/4	1/4	4/4
10 ⁻⁵				2/4				1/4
10 ⁻⁶								1/4
10 ⁻⁷								0/3
10 ⁻⁸								0/3

Unheated control:

Infectivity: 10^{5.2} MID₅₀/ml.

Protein: 42.5 mg/ml.

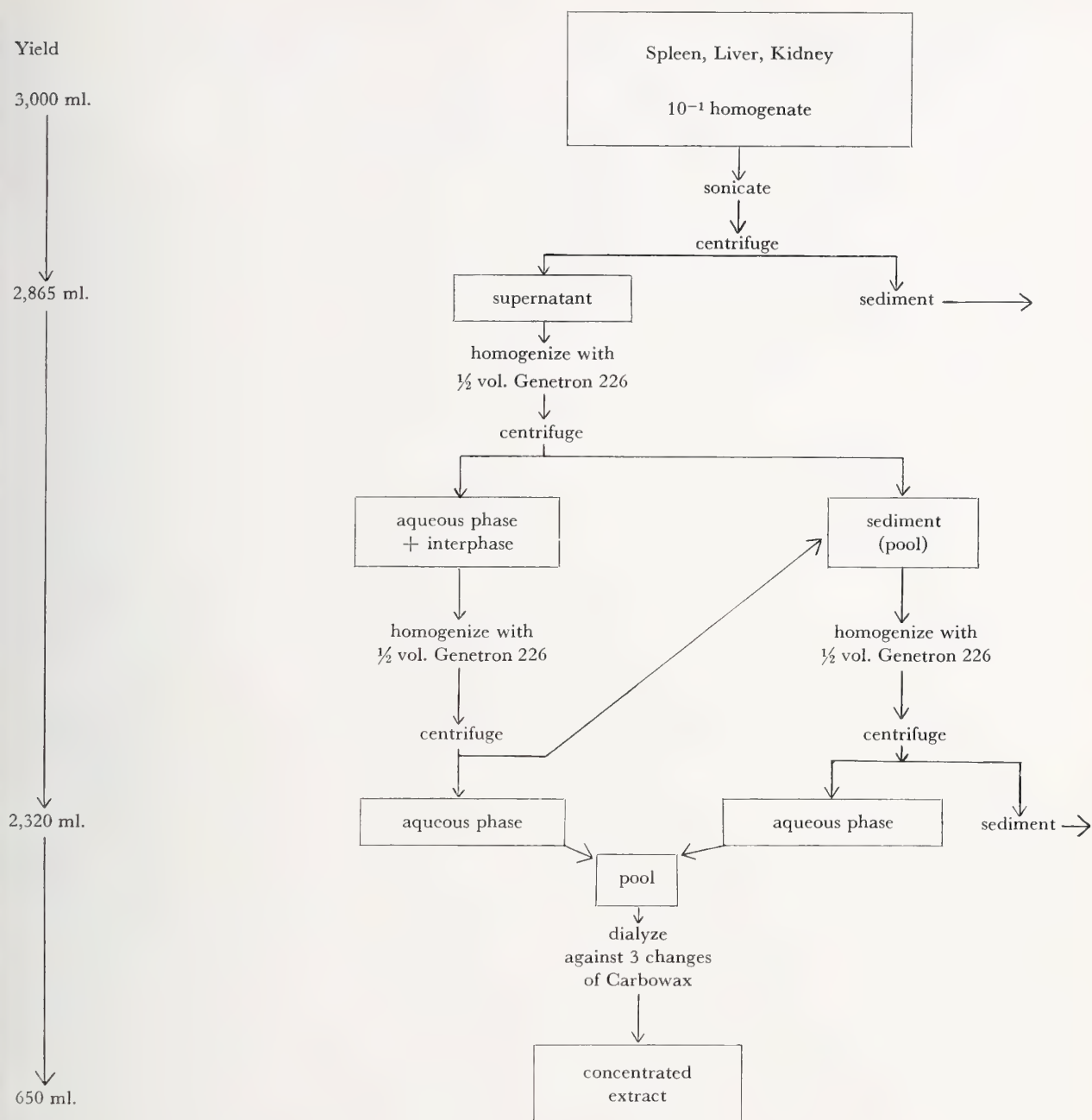
¹ Number affected / number injected.

al. (9) Epstein (10) has utilized this technique for purifying vaccinia virus and Rous sarcoma virus. The effect of the fluorocarbon treatment is not yet fully understood; but it probably acts as a selective denaturing agent, destroying and collecting various cell components and proteins into the fluorocarbon phase.

Our flow sheet is shown in table III. Three hundred gm. of liver, spleen, and kidneys were harvested from mink with experimental AD in the advanced stages of the malady. The organs were pooled, minced and then homogenized in a Waring blender with enough phosphate buffered saline (pH 7.2) to give a 10-percent weight/volume tissue suspension. Further homogenization of the tissue suspension was accomplished by sonication with a Branson sonifier. The homogenate was centrifuged for 30 minutes at 8,000 g., and the opalescent reddish supernatant fluid was extracted with 1/2 volume of Genetron 226¹ (CF₂Cl-CCl₂F, density 1.6). The extraction was carried out in the cold (icebath +5° C.) by agitation of the

¹ Genesolv-D, General Chemical Division, Morristown, N.J.

TABLE III.—Procedure for Fluorocarbon Extraction of Mink Tissue



Genetron tissue mixture for 4 minutes at high speed in a Waring blender. The homogenate was separated into an aqueous, intermediate and fluorocarbon phase by centrifugation for 15 minutes at 8,000 g.

Preliminary studies indicated that AD infectivity remained in the pooled aqueous and intermediate phases. The latter two fractions were extracted a second time with $\frac{1}{2}$ volume of Genetron. After separation by

centrifugal force, the aqueous phase was saved. The sediments of both extraction steps were recycled in another fluorocarbon treatment. Finally, the aqueous phases were pooled and dialyzed against three changes of Carbowax.² The latter procedure resulted in a 4-

² Carbowax 4000, Union Carbide Chemicals Co., New York, N.Y.

fold concentration of the extract. The clear extract had a light reddish-brown color.

As revealed in table IV, this extraction procedure resulted in an 8-fold reduction of the protein content, without apparent loss of infectivity. This extract was used in the tests described below.

TABLE IV.—Infectivity Assay of Fluorocarbon Extracts

	Experiment I		Experiment II	
	Protein mg./ml.	Infectivity log MID ₅₀ /ml.	Protein mg./ml.	Infectivity log MID ₅₀ /ml.
Crude:	43.7	5.8	46.8	>5.3
1. Fluorocarbon	11.3	>5.3		
2. Fluorocarbon	6.1	>5.3	5.6	>5.3
3. Fluorocarbon	4.8	>5.3		

Resistance to Thermal Inactivation

The resistance of the AD agent to thermal inactivation was tested by heating several tubes, each containing 5 ml. AD extract or crude tissue suspension in a waterbath. The temperature within the tubes obtained after a preheating period of 5 minutes was 90° to 95° C.

Samples were taken at intervals and, after rapid cooling, injected into test mink. The partially purified extract was biologically inactive after heating for 15 minutes or longer. On the other hand, the crude tissue suspensions were still infectious at 15 minutes but not after 30 minutes (tables Va and Vb).

TABLE Va.—Partially Purified A.D. Extracts. Resistance to Heat Inactivation (90° to 95° C.)

Inoculum	Heated for						
	15'	30'	45'	60'	90'	120'	180'
	Number affected/number injected						
1 ml. concentrated extract.	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Infectivity: $>10^{5.3}$ MID₅₀/ml., $<10^{5.8}$ MID₅₀/ml.
Protein: 5.6 mg./ml.

The accelerated inactivation of the partially purified preparation might be a chance observation or could, perhaps, have been due to a damaging effect of the extraction procedure on the agent. Conversely, the apparent stability of the AD agent in crude suspensions

TABLE Vb.—Crude A.D. Tissue Suspension. Resistance to Heat Inactivation (90° to 95° C.)

Inoculum	Heated for	
	15'	30'
	Number affected/number injected	
1 ml. crude tissue homogenate.	4/4	0/4

Infectivity: $>10^{5.3}$ MID₅₀/ml., $<10^{5.8}$ MID₅₀/ml.
Protein: 46.8 mg./ml.

(table Vb) was perhaps due to a stabilizing effect of impurities.

Resistance to Chemical Inactivation

(a) *0.4 Percent Formalin.*—Samples of AD extract and crude tissue suspension were treated with 0.4 percent formalin and kept at 37° C. for periods varying from 12 hours to 96 hours. The AD extract was rendered noninfectious after treatment for 12 hours (table VIa). However, five of six mink were infected with a crude tissue suspension treated similarly for 12 hours (table Vlb). Since proteins are known to bind formalin, the increased protein content in the crude suspension may explain the differences in formalin resistance.

TABLE VIa.—Partially Purified A.D. Extracts. Resistance to 0.4 Percent Formalin

Inoculum	Treatment with formalin at 37° C. for							
	12 ^h	24 ^h	36 ^h	48 ^h	60 ^h	72 ^h	84 ^h	96 ^h
	Number affected/number injected							
1 ml. concentrated extract	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Infectivity: $>10^{5.3}$ MID₅₀/ml., $<10^{5.8}$ MID₅₀/ml.
Protein: 5.6 mg./ml.

(b) *Other Chemicals.*—In another experiment six tubes each containing 5 ml. of AD extract were treated with one of the following chemicals: sodium hydroxide, hydrochloric acid, urea, Lugol's iodine, ethyl ether, or sodium desoxycholate. The final concentrations of these chemicals in the test suspensions are shown in Table VII. All samples were shaken thoroughly and allowed to stand at room temperature for 2 hours prior to inoculation.

TABLE VIIb.—Crude A.D. Tissue Suspension.
Resistance to 0.4 Percent Formalin

Inoculum	Treatment with formalin at 37° C. for
	12 ^h
Number affected/number injected	
1 ml. crude tissue homogenate	5/6

Infectivity: $>10^{5.3}$ MID₅₀/ml., $<10^{5.8}$ MID₅₀/ml.
Protein: 46.8 mg./ml.

As shown in table VII the AD agent was readily inactivated by strong bases and acids, as well as 0.5 percent solutions of Lugol's iodine. We assume that 4 N urea was partially effective in destroying this agent as judged by the slow development of hypergammaglobulinemia in two animals. A third animal, after showing a questionable IAT, proved positive only at histologic examination. We believe the effect of urea on the AD agent deserves reinvestigation. Under the conditions of these tests, treatment with ether and sodium desoxycholate did not destroy AD infectivity.

TABLE VII.—Resistance to Chemical Inactivation

Treatment*	Number affected/number injected
0.05N NaOH	0/4
0.05N HCl	0/4
4N urea	3/4
0.5 percent iodine	0/4
½ vol. ether	4/4
0.5 percent sodium desoxycholate	4/4

Infectivity: $>10^{5.3}$ MID₅₀/ml., $<10^{5.8}$ MID₅₀/ml.
Protein: 5.6 mg./ml.

*2 hours at room temperature.

Resistance to Enzymatic Treatment

In the following experiment, the resistance of the AD agent to the action of pancreatic DNase, RNase and the proteolytic enzymes pepsin, trypsin, papain, and pronase were tested. One ml. of a freshly prepared enzyme stock solution was added to 9 ml. samples of partially purified AD extract. The test samples were mixed thoroughly and then incubated for 2 hours at 37° C. Each of four mink was injected intramuscularly with 1 ml. of the test material. As shown in table VIII the agent was resistant to treatment with nucleases and all proteases tested.

TABLE VIII.—Resistance to Enzymatic Inactivation

Treatment ¹	Number affected/number injected
0.1 percent DNase	4/4
0.1 percent RNase	4/4
0.1 percent Pepsin	4/4
0.1 percent Trypsin	4/4
0.1 percent Papain	4/4
0.1 percent Pronase	4/4
None	0/25

Infectivity: $>10^{5.3}$ MID₅₀/ml., $<10^{5.8}$ MID₅₀/ml.
Protein: 5.6 mg./ml.

¹ 2 hours at 37°/C.

In another experiment (tables IXa and IXb) the combined effect of proteases (pepsin, trypsin, or pronase) and nucleases was tested. After pretreatment for 1 hour at 37° C. the action of the proteolytic enzymes was reduced by addition of 1 percent bovine serum to each tube. Immediately thereafter 0.1 percent DNase or RNase was added and the digestion continued for another hour. No detectable AD agent inactivation was observed.

TABLE IXa.—Resistance to Enzymatic Inactivation
(Proteases + DNase)

Treatment	Number affected/number injected
Pepsin + DNase	4/4
Trypsin + DNase	4/4
Pronase + DNase	4/4

TABLE IXb.—Resistance to Enzymatic Inactivation
(Proteases + RNase)

Treatment	Number affected/number injected
Pepsin + RNase	4/4
Trypsin + RNase	4/4
Pronase + RNase	4/4

Infectivity: $>10^{5.3}$ MID₅₀/ml., $<10^{5.8}$ MID₅₀/ml.
Protein: 5.6 mg./ml.

DISCUSSION

According to these data the partially purified AD agent shows a surprising stability against the action of proteolytic enzymes and nucleases. In contrast, it is

readily inactivated by boiling or treatment with strong acids, bases, or iodine. It is noteworthy that the crude preparation exhibited greater stability towards heat (90° to 95° C.) and 0.4 percent formalin. We believe that the discrepancy can be explained by a possible stabilizing effect of impurities on the AD agent.

The results reveal that the protein content of tissue preparations can be reduced considerably by a simple fluorocarbon extraction procedure without detectable effect on the biological activity of the agent. The use of such extracts will be helpful in studying the role of a viral agent in the pathogenesis of AD of mink.

It is also apparent that in comparison to the overwhelming amount of impurities still present in such extracts, the total mass of virus must be small—even insignificant. It is anticipated that further work will yield virus preparations which are suitable for conclusive chemical and biophysical analysis of the causal agent.

SUMMARY

Aleutian disease virus was extracted with fluorocarbon (Genetron 226) from infected mink tissues.

The infectivity of these partially purified extracts was destroyed by heat (90° C. for 15 minutes), 0.4 percent formalin (12 hours at 37° C.), 0.05 N HCL (2 hours at 25° C.), 0.05 N NaOH (2 hours at 25° C.), and 0.5 percent Lugol's iodine (2 hours at 25° C.).

The virus was ether and desoxycholate resistant. It was not inactivated by proteolytic enzymes (pepsin, trypsin, papain, pronase) or DNase or RNase.

In crude tissue suspensions the AD virus exhibits a greater stability towards heat (90° to 95° C.) and 0.4 percent formalin.

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DISCUSSION

KENYON: I would think that it would be a good idea to utilize serum or urine fractionation procedures, since the methods of purification of serum proteins are at a more advanced level than those with cellular and subcellular fractionations. Has anyone tried to obtain the agent from other biological fluids?

BURGER: The problem is, I suppose, getting enough serum. Extraction of mink tissue is quite simple and can be done in a day or so, and I think that the partially purified material as presented here probably gives as good a yield as the other would do.

BROTHERSTON: I would like to mention scrapie in this context. The scrapie agent is so firmly attached to particles that you cannot reduce the protein content without reducing the titer. This really makes concentration difficult. I would think that even in studies such as this it would be almost useless to look at the concentrate under the electron microscope because even 10^5 concentration is too low for you to be able to see enough to pick up an agent.

PATTISON: I was greatly intrigued about the RNase and DNase treatment. How do you interpret this, for, presumably, if it is a virus, it contains either RNA or DNA. What weight do you put on a test of this kind?

BURGER: If you get inactivation with one nuclease, but not the other one, it gives an indication of the actual nucleic acid type. We did not get inactivation with

either type and I think this tells us a little bit about the stability of the agent; presumably, a protein coat protects the virus and does not allow the virion to be penetrated by a nuclease. We know, from other agents, that this can be the case.

CASALS: Do you have any evidence of a serological reaction of some kind? You know, it is awfully hard to work with a virus which you do not see and which you cannot react with in any way. The mink may not develop antibodies, but with titers of the order of 10^{-6} or 10^{-7} , if you inoculate rabbits, guinea pigs, or any animal, you should, presumably, get an antiserum which then could neutralize your virus. If you could get a positive reaction of that sort, then it would be quite an achievement.

BURGER: This was one of the reasons we did this experiment because if you want to try to go to another species to get antiserum, you first have to clear your inoculum from all unnecessary proteins.

LEADER: I think that with pools of fluorocarbon extracted material we now have an antigen that is

much better and we can work with this to produce neutralizing antibody and also fluorescent conjugates to stain for antigen in the cells.

FIELD: This epithelial proliferation in the bile ducts, do you see mitoses there?

LEADER: The cells must be multiplying, but I have not seen mitoses.

HADLOW: I too have been impressed by the lack of mitotic figures in all this proliferation.

GIBBS: Dr. Hadlow, I believe that you have done some filtrations for size. Did you or are you planning to do this?

HADLOW: We did a limited study only, using millipore and gradacol membranes. I think we started with a 10^{-2} preparation of our working pool and the experiment was done in sapphire female kits. Filtrates that passed 100 millimicron gradacol membranes and those that passed 50 millimicron millipore membranes proved infectious for five out of five mink in each case.

The Comparative Aspects of Aleutian Mink Disease (AD)

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INTRODUCTION

The fact that AD in mink is an infectious disease caused by a filterable agent (1, 2, 3) gives rise to the possibility that other species might be involved. Since urine has been shown to contain the agent (4), contact with this excretion could be such a means. The vicious nature of the mink results in urine being voided, splashed about, and the animal biting when being handled. Thus, the requisites for transmission to man are present. The possibility became a serious consideration in 1963 when Chapman and Jimenez (5) reported the death of a mink rancher following a lengthy disease. The clinical picture and the autopsy report contained factors suggesting AD, and the authors concluded AD as the cause of death. This was followed by the paper of White et al. (6) who reported the finding of abnormal bodies in the cytoplasm of lymphocytes from the peripheral blood of 6 of 12 mink ranchers; these ranchers were in good health at the time of the study. White and coworkers (6) felt that they saw this phenomenon in the smear of the peripheral blood of the patient of Chapman and Jimenez and they considered this change in their patients to be associated with mink ranching. Similar bodies have been reported by Padgett et al. (7) in the blood of Aleutian mink; these authors reported the situation to be a musteline type of the Chediak-Higaski syndrome. Now a third situation, as yet unreported, has developed in a young man originally thought to be ill of Hodgkin's disease. The attending physicians are now entertaining the possibility of AD. To use White et al.'s

title, "Aleutian Mink Disease in Man, Fact or Fancy?" seems appropriate to justify further consideration of the zoonotic possibilities of this malady.

MATERIALS

The materials to be discussed are histologic sections from the autopsy of the Chapman and Jimenez case, a lymph node biopsy from a patient (R.T.) under the care of one of us (B.H.D.), as well as mink which were experimentally infected with the disease and field cases.

DISCUSSION

Certain disadvantages are inherent in such a comparison, namely, (1) a disease may produce somewhat different lesions in an unnatural host; (2) organs may vary from species to species; and (3) the tissues available from the human cases are few in number.

A concept of the nature of AD becomes necessary before a direct comparison is made. We believe AD to be a chronic autoimmune disease progressing inexorably to death. The malady is characterized by a striking hypergammaglobulinemia which is directly proportional to an equally striking proliferation of plasma cells in such organs as exemplified by the liver and the kidney. These plasma cells are by and large mature and seem to have a multicentric and simultaneous origin; it is plausible to attribute their rise directly from the reticuloendothelial system (RES) on the basis of distribution (3). It is believed that the plasma cell proliferation is stimulated by a viral-like

agent, which upon entering the body is monitored by the RES (8), which in turn commences the plasma cell production. The nature of the disease is proliferative in the sense of inflammation but not in the sense of neoplasia. One example will suffice: in the liver the plasma cells invariably arrange themselves about the periportal area and are accompanied by bile duct proliferation (9, 10).

The possibility that AD is similar to multiple myeloma of man has been proposed (11). Multiple myeloma is a specific entity in man with a fairly characteristic if not pathognomonic lesion picture. The so-called "punched out" areas are the result of the neoplastic process in the bone. This never occurs in mink. Another point is that multiple myeloma is a malignancy sometimes referred to as plasmacytoma whereas AD appears inflammatory in character. Furthermore, immunoelectrophoresis showed large amounts of gamma₂-7S components in urine of AD affected animals. Analyses of these urinary proteins indicated they were from 1S to 2S and had electrophoretic mobilities of gamma and beta globulins (12, 13).

For the first comparison an axillary lymph node from a 19-year-old man (R.T.) who had been bitten several times by AD affected mink will be considered. A cervical lymph node was removed in September 1963 and Hodgkin's disease was the histologic diagnosis. Nitrogen mustard appeared to improve his condition. In May 1964 he began to experience daily febrile episodes not typical of the Pel-Epstein pattern of Hodgkin's disease. Furthermore, he developed a hypergammaglobulinemia of 3.64 gms. Some physicians are now reluctant to consider this disease as Hodgkin's. The lymph node had no definite cortical or medullary areas; it presented a study of monotony with follicular-like structures. Yet it was intact and showed no evidence of breaking through the capsule, which is also true in AD affected mink. A higher power revealed large, pale cells (fig. 6) amid the background of the far more numerous chromatic ones. These were considered to be large macrophage cells, not the Reed-Sternberg type. But on closer examination, one was struck by the plasma cell numbers (fig. 3). These were doubtless plasma cells as illustrated by the eccentric nucleus with its chromatin clump arranged in the classical wheel spoke fashion.

The next comparison will be a lymph node of the Chapman and Jimenez (5) patient. In short, this man had a chronic illness marked by hepatosplenomegaly, lymphadenopathy, and above all, hypergammaglobulinemia (4.6 gms. per 100 ml.). He too had

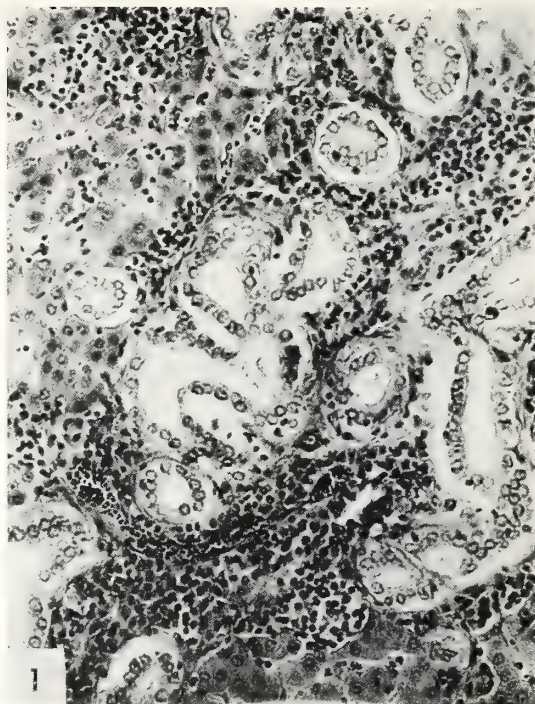


FIGURE 1.—Liver from a mink with Aleutian disease showing bile duct proliferation and periportal plasma cell infiltration. H. & E. stain. $\times 182$

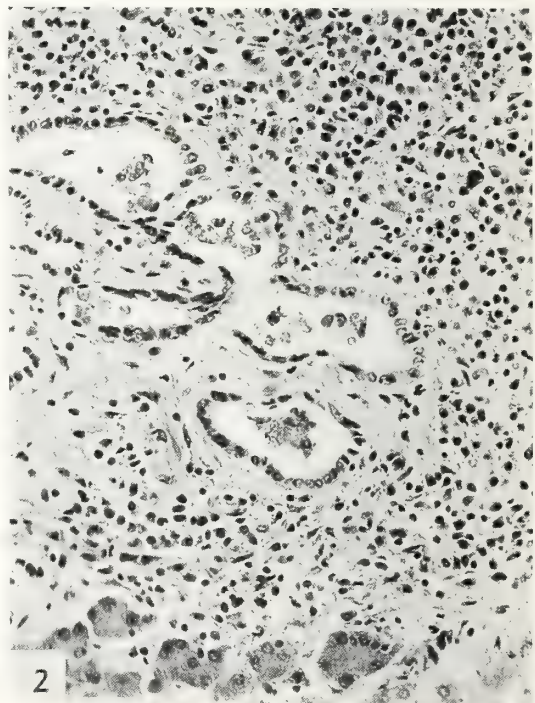


FIGURE 2.—Liver from a man diagnosed as Aleutian disease (5) showing the same type of lesion as in figure 1. H. & E. stain. $\times 182$

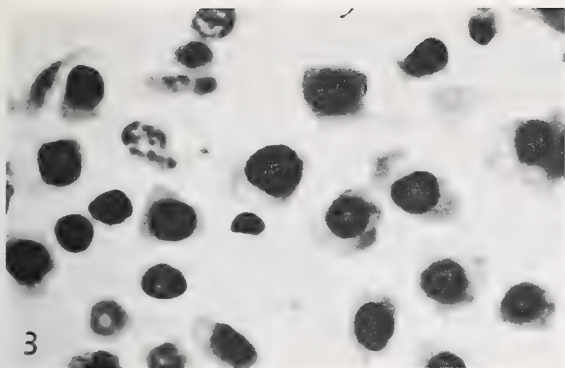


FIGURE 3.—Plasma cells from the lymph node of a mink with Aleutian disease. The cytoplasm is showing a lacy pattern suggesting Russell body formation. H. & E. stain. $\times 480$

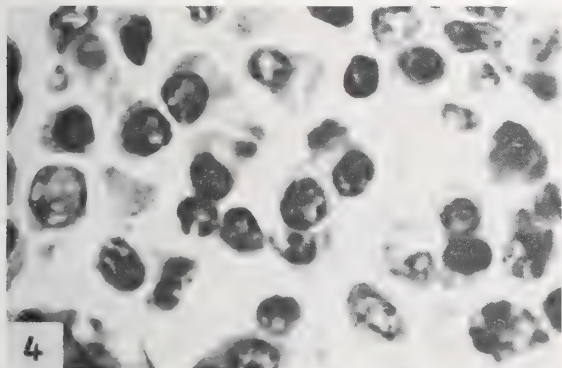


FIGURE 4.—Plasma cells from the lymph node of R.T. These are becoming vacuolated as in figure 3. H. & E. stain. $\times 480$

been repeatedly bitten by sick mink. The lymph node integrity was abolished by sheets of plasma cells and vague follicles (fig. 7) but as in the previous case the capsule was intact. When compared to a mink lymph node there appeared to be a great disparity as the architecture seemed well preserved in some mink. Yet in other mink the lymph node appeared identical (fig. 5) with the exception that the large macrophage was not as prominent. By and large the impression was that the human lymph nodes were more altered than those of the mink. When higher powers were used then the plasma cells within the node began to appear (fig. 3) and the plasma cell numbers seemed about the same.

In common were: (1) marked plasmacytosis; (2) intact capsule. Not consistent was the fact that lymphadenopathy was not a common finding in AD affected mink.

In the mink the bone marrow contained many plasma cells usually in a diffuse distribution. There were no other changes, as trabeculae, fat, and hematopoiesis seemed normal. The Chapman and Jimenez

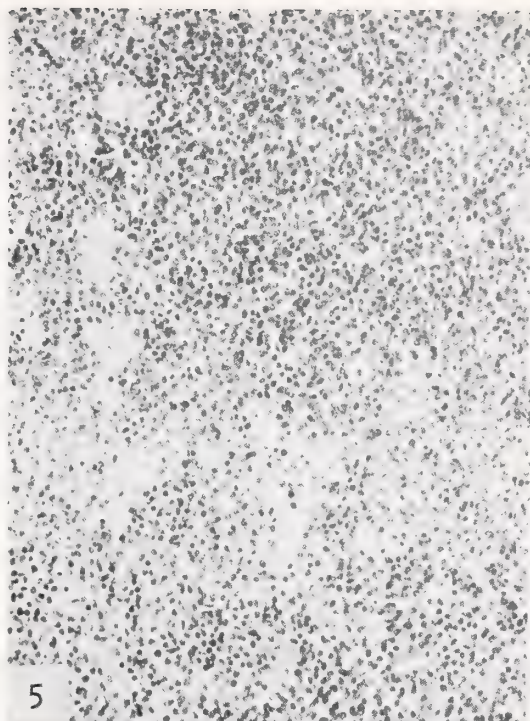


FIGURE 5.—Lymph node from a mink with Aleutian disease showing rather uniform sheets of cells which are a mixture of plasma cells and lymphocytes. H. & E. stain. $\times 75$

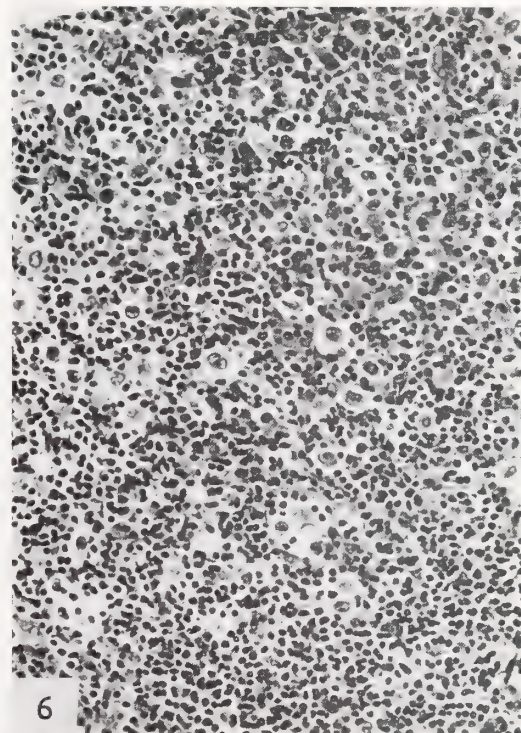


FIGURE 6.—Lymph node from a man diagnosed as Hodgkin's disease and also as Aleutian disease. Large macrophages are seen on a background of smaller and more chromatic types, which are almost all plasma cells. H. & E. stain. $\times 182$

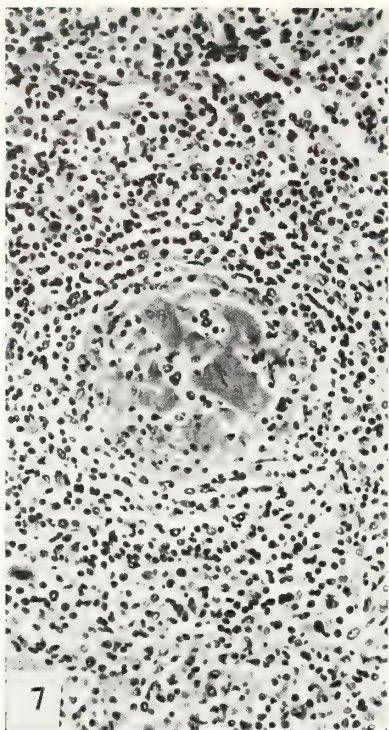


FIGURE 7.—Spleen from a man diagnosed as Aleutian disease (5) showing amyloid surrounded by small cells which on higher power are almost all plasma cells. H. & E. stain. $\times 182$

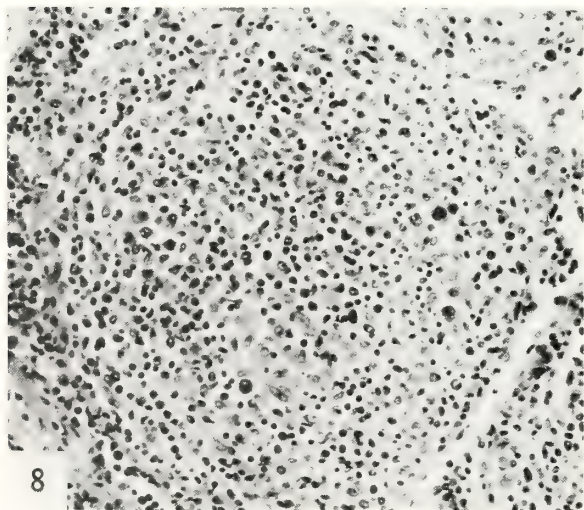


FIGURE 8.—Same spleen as in figure 7 showing the plasma cell granuloma. H. & E. stain. $\times 182$

patient had bone marrow which was only moderately hyperplastic according to their interpretation. There were far more than the expected 3 percent plasma cells, in fact the similarity between the species here was considerable but only because the deviation from the normal was not great.

The spleen in AD affected mink was remarkably hypertrophied—5 times normal size being common. The architecture was fairly rich in plasma cells particularly the red pulp, but the follicles were often intact. One thing which would impress the histologist unfamiliar with mink spleen is the large numbers of megakaryocytes ordinarily present. The spleen of the Chapman-Jimenez patient weighed 1,400 gms. and was marked by an increase of inflammatory cells which were present most prominently within the follicular-like structures which were considered to be plasma cell granulomas (fig. 7). The picture was one of proliferation to obscure normal landmarks. In one area amyloid was noted (fig. 8).

The plasmacytosis was again the common feature. The changes in the human patient were more impressive than those seen in the mink, which although rich in plasma cells tended to maintain architectural landmarks.

In the liver the comparison could be termed identical. Periportal infiltrates of plasma cells and bile duct proliferation were seen in the human cases and mimicked precisely by the mink (figs. 1, 2).

One lesion that was not seen in the human tissue was the periarteritis seen in considered terminal cases of the mink. The significance of this lesion can be overrated in a comparison such as this; the lesion occurs at about 12 percent in field cases and rarely in experimental cases.

From the standpoint of comparative histopathology, the lesions in the two human beings were similar to those ordinarily seen in the mink. The striking hypergammaglobulinemia along with splenomegaly and hepatomegaly were also common features.

SUMMARY AND CONCLUSIONS

A limited number of human tissues, namely, lymph node (Case 1), lymph node, spleen, bone marrow, and liver (Case 2) from patients thought to have had Aleutian mink disease (AD) were compared with AD affected mink tissue. The hepatic periportal infiltrates of plasma cells with bile duct proliferation was common to both species. In the spleen and lymph nodes the plasmacytosis was more marked than in the mink. From the standpoint of comparative pathology, there is a considerable similarity between AD in mink and the diseases considered to be AD in the human patients.

ADDENDUM

The possibility of Aleutian disease (AD) existing as a latent infection in mink ranchers may be

greater than previously realized. This is based on the fact that the ferrets can be infected and yet not show any detectable signs of illness. Evidence of AD infection in ferrets is established by transmitting AD back into mink with tissue suspensions of ferret origin. These have been obtained as late as 60 days postinoculation (AD infective tissue extracts of mink origin) and have still been highly infectious in mink.

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DISCUSSION

KIBRICK: I wonder whether it would be worthwhile, in the absence of a serologic test of any kind, to heat inactivate some of the material and see if the particular patient is skin-test positive. If he were, then I suppose one could similarly test other mink ranchers and other people for hypersensitivity to this antigen.

WILLIAMS: I have some data pertinent to this particular question. We tested 83 mink ranchers from all over Wisconsin and Minnesota at a mink ranchers' convention. We studied quantitative levels of 7-S gamma globulin, beta 2-A, and macroglobulin by a micro diffusion technique and in none of these individuals were we able to find any characteristic abnormalities in the immunoglobulins. So, if this disease occurs in man, perhaps it occurs in a strain of man as rare as the homozygous Aleutian mink.

KIBRICK: There is another technic not yet mentioned which could be employed either to detect chronic infection with this agent or to determine whether infection may have occurred in the past. This technic has advantages over the use of a skin test since it avoids introduction of antigen into test subjects and, therefore, carries no risk. It has been shown with tuberculin and other antigens that human blood lymphocytes in culture will proliferate under stimulus of an antigen to which the donor is sensitive. A patient with supposed Aleutian mink disease is currently available for study. It would be simple to determine whether cultured lymphocytes from this patient respond to Aleutian mink disease antigen (i.e., a tissue suspension from infected mink) by a proliferative response. Should such a response be obtained, lymphocytes from suspects could be similarly tested for evidence of hypersensitization to this antigen.

Enhancement of Aleutian Disease Plasmacytosis by Immunoglobulins from Infected Mink¹

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Aleutian disease (AD) of mink has been considered to be of viral origin (1) and described as having certain features in common with the autoimmune diseases of man (2). One of the more prominent features is the slow, steady progression of the disease accompanied by plasmacytosis and a greatly increased serum level of 7S gamma globulins (3).

The immunologic competency of the proteins accounting for the characteristic AD hypergamma-globulinemia as antibodies, in the classical sense or as autoantibodies, has not been established nor experimentally related to the pathogenesis of AD. No evidence of neutralizing activity was found in experiments performed by Gorham et al. (4) in which they sought reduction in infectivity of infective extracts added to hypergamma-globulinemic sera from infected mink. The fact that AD can be transmitted in the presence of globulins, chemically classed as immunoglobulins, has not eliminated the possibility that these proteins have antibody character directed at determinants of the etiologic agent. On the other hand, the correlation of increased serum gamma globulin levels with the presence of lesions does not necessarily implicate these protein components in the pathogenesis of lesions similar to those resulting from autoimmune reactions.

Since no apparent benefit has been observed as the result of the hypergamma-globulinemia, preliminary experiments described in this report were performed in an attempt: (1) to demonstrate a disease-enhancing

activity by euglobulins from infected mink and (2) to ascertain the effect of these proteins on the disease-limiting function of "sensitized" macrophages and monocytes.

MATERIALS AND METHODS

The experimental design utilized 63 female 1-year-old pastel mink, heterozygous (Aa) for the Aleutian gene, which were divided into 4 main groups with their appropriate controls. Group I was inoculated with infective tissue homogenate; Group II received the same homogenate, incubated with euglobulins from infected mink; Group III received tissue homogenate incubated with cells from peritoneal washings; and Group IV received tissue homogenate, peritoneal cells, and euglobulins.

The infective material used throughout this study was obtained from standard dark mink having histopathological lesions characteristic of AD. Kidney tissue (38 gm.) obtained from mink with lesions differing in severity was homogenized in 150 ml. of phosphate buffered isosaline (pH 7.6), subjected to sonorization in a Raytheon Sonic Oscillator, 250-W, 10-Kc for 90 seconds and then sedimented for 45 minutes at 10,000 \times g. An aliquot of the supernatant fluid was diluted tenfold with the same buffer and filtered on a 450 m μ millipore filter to form the stock tissue homogenate (TH). When 1 ml. (containing 0.035 mg. protein/ml.) of this stock solution was inoculated intraperitoneally in a series of 1-year-old mink heterozygous for the Aleutian gene (Aa), 95 percent became infected, with a 40 percent mortality at 100 days post inoculation.

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The euglobulin preparation used throughout was prepared from AD sera by ammonium sulfate fractionation (Smith (5) and Kekwick (6)) followed by reprecipitation from distilled water. Some of the properties of these proteins and the details of preparation have been previously described (7). The average gamma globulin concentration was 49 percent in the individual serum pooled for protein preparation. To test the immunological properties, the euglobulins were dissolved in phosphate buffered isosaline (pH 7.6) at a 1 percent protein concentration and then filtered on a 10 m μ millipore filter to form the stock euglobulin solution (Eug.). After equal volumes of TH and Eug. were incubated for 4 hours at 37° C. followed by refiltering on 450 m μ millipore filter, 2 ml. of this protein solution-tissue extract mixture was made to 3 ml. volume with phosphate buffered saline and injected (Group II).

The structure of the experimental design was such that the first lot of mink inoculated (Group I) with 1 ml. of TH (adjusted to 3 ml. volume with phosphate buffered saline) would at 30 days provide a source of "sensitized" macrophages in peritoneal washings for succeeding experiments. The peritoneal washings were obtained from two mink by peritoneal lavage under ether anesthesia with 100 ml. of osmolor sucrose (70 percent recovery of perfused volume). In each case the cells obtained were sedimented from the sucrose solution and resedimented three times from 200 ml. phosphate buffered isosaline, then finally suspended in 20 ml. of the same buffer to form the stock peritoneal cells (PC) containing approximately 15,000 WBC/ml. with macrophages accounting for about 15 percent of the cells. The cell suspensions were inoculated into mink (Groups III and IV) as follows: in Group III, an equal volume of TH and PC suspension were incubated at 37° C. for 1 hour, followed by intraperitoneal inoculation (2 ml. cell suspension and 1 ml. buffer per mink); Group IV received 3 ml. intraperitoneal inoculums of an incubated mixture containing equal volumes PC, TH, and Eug. The details of the experimental plan, numbers of mink in each of the four groups, and their controls may be seen in table I.

At approximately 30 and 45 day intervals blood was obtained by cardiac puncture for serum electrophoretic analyses. Paper electrophoretic analyses were conducted in the usual manner as previously applied to mink serum (3, 7). All surviving mink were killed and subjected to postmortem examination at 100 days. Tissue sections were taken from kidney, liver, spleen,

TABLE I.—Effect of Euglobulin on AD Transmission

Group	Number of mink	Inoculum	Percent infected	Percent mortality
Group I	10	AD tissue extract	90	40
Group II	10	AD tissue extract and euglobulin	90	² 50
Controls ¹	4	Uninoculated mink	0	0
	10	Euglobulin	0	0
Group III	10	AD tissue extract plus peritoneal cells.	40	² 20
Group IV	10	AD tissue extract, peritoneal cells, and euglobulin	100	20
Controls ¹	3	Uninoculated	0	0
	6	Peritoneal cells	17	² 0

¹ In addition, 30 uninoculated mink were held as controls in conjunction with another experiment and had no detectable clinical signs of AD.

² The death of one mink as the result of cardiac puncture is not included in percent value for the group designated.

and sternum, fixed in neutral buffered formalin, and stained with hematoxylin-eosin for histopathologic analyses.

RESULTS

The addition of 10 m μ filtrates of euglobulin solutions to infective tissue homogenates (Group II) produced an increased mortality (figs. 1 and 2) over that observed in Group I which received tissue homogenate alone and secondly, more pronounced signs of AD—polydipsia, cachexia, melaena, and anorexia. The most obvious early sign of AD was reduced food consumption after 20 days and emaciation by the 45th day.

Histologically, there were some indications of a higher proportion of lymphocytes in the infiltrates than plasma cells as compared to the ratio of cell types observed in Group I. The lower serum gamma globulin values of mink in Group II also indicate a limited plasma cell proliferation. However, since more mink died in the early stages of the experiment in Group II, the differences in lesions and average gamma globulin levels (fig. 3) seen in Groups I and II may not be representative of the treatment for Group II.

The mink which received tissue homogenates incubated with peritoneal cells had a lower mortality due to Aleutian disease than either Group I or Group II and also a lower incidence of apparent infection (fig. 4). The characteristic morphological changes used as criteria of AD infection were lymphocytic and plasma-

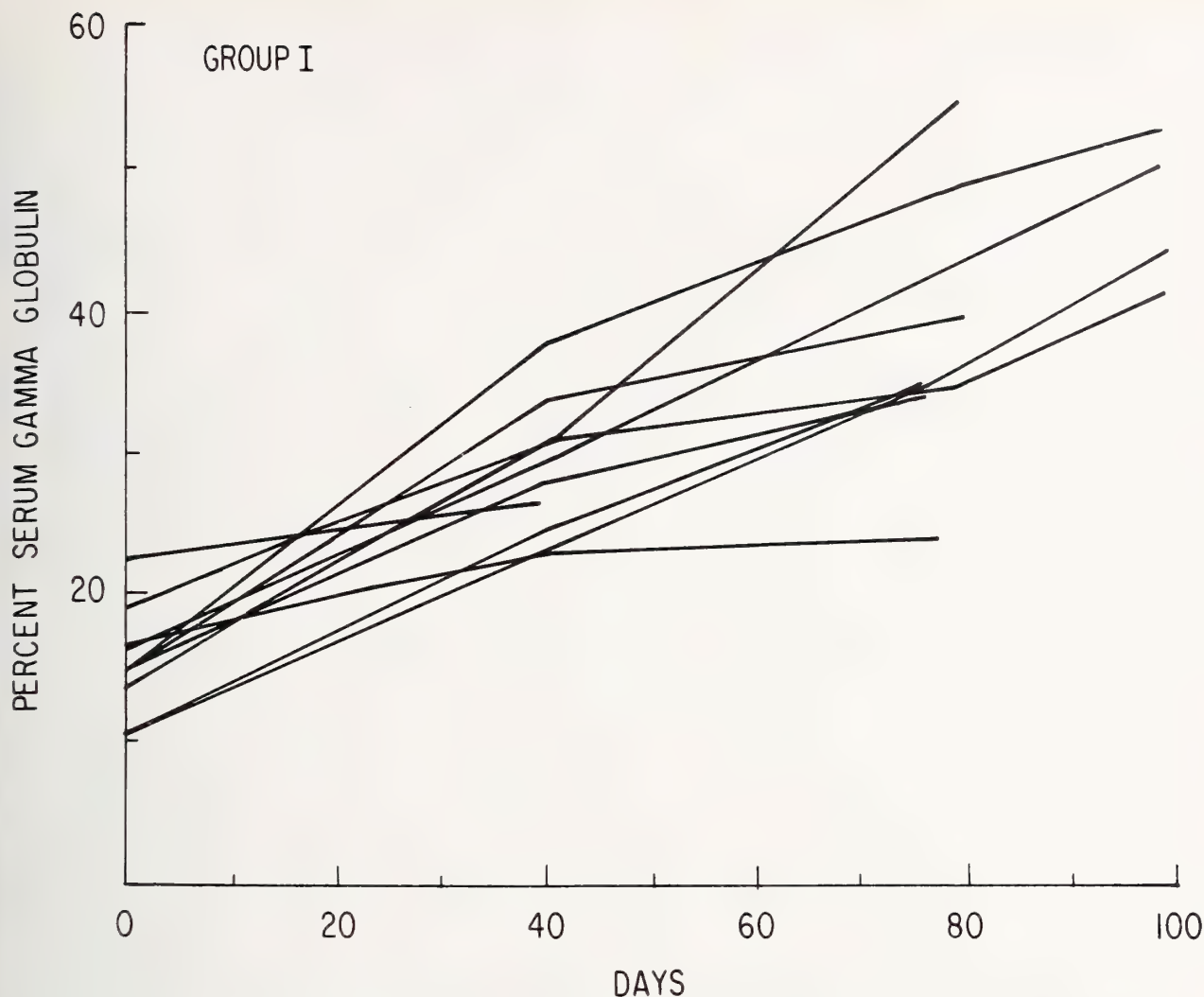


FIGURE 1.—The values plotted are percent gamma globulin of total serum protein from mink receiving AD tissue homogenate. Death is indicated by termination of curve at the last date of serum analysis prior to death.

cellular infiltrates in the renal cortex, portal triads, spleen, lymph nodes, and bone marrow. Hyperplasia and dilation of the bile ducts were commonly associated with the plasma cell infiltrates in the portal triads. Since serum gamma globulin levels closely parallel the degree of plasma infiltration (3), considerable reliance was placed on the electrophoretic characterization of serum proteins. The data in table I shows the percent mortality at the termination of the experiment as 20 percent for Group III as opposed to 40 percent for Group I and 50 percent for Group II. Similarly, the average serum gamma globulin level of Group III was lower than in Group II. This represented a lower degree of cellular infiltration rather than a shift in the lymphocyte/plasma cell ratio. Only one control mink which received washed peritoneal cells showed an apparent infection.

The mink in Group IV like Group III (figs. 4 and 5) had a low death rate, but unlike Group III, all had massive infiltrates of higher proportions of plasma cells than was seen in the other groups. The only difference in the treatment of these last two groups was the incorporation of euglobulin with the infective homogenate and peritoneal cells.

DISCUSSION

The fact that Gorham et al. (4) did not observe a classical antibody response when sera from infected mink are combined with infective tissue extracts and the observation that serum alone from mink with AD produced the disease, has prompted a consideration of the role of AD "immunoglobulins" with respect to disease enhancement. Although disease enhancement by the

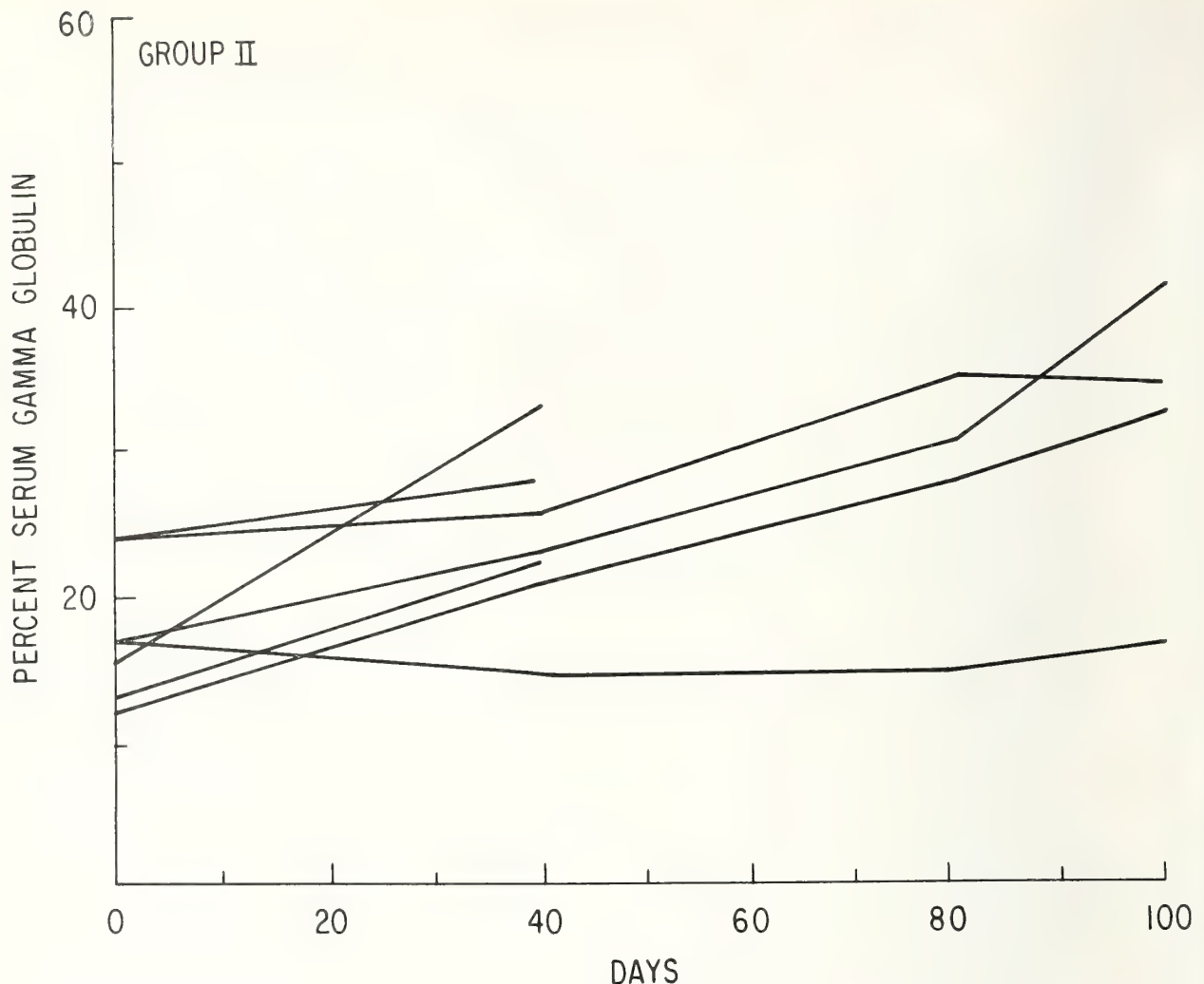


FIGURE 2.—The values plotted are percent gamma globulin of total serum proteins from mink receiving AD tissue homogenate and euglobulin from mink with hypergammaglobulinemia. Death is indicated by termination of the curve at the last date of serum analysis prior to death.

vastly increased gamma globulin concentrations may be associated with a number of factors, including non-immunological biophysical properties of the proteins, those sought here are considered analogous to tumor-enhancing cytolytic antibodies or those of an autoimmune nature.

When euglobulin preparations from hypergammaglobulinemic sera were inoculated intraperitoneally in control mink subsequent to a filtration process which would have removed particles in the size range of the AD "virus," 5 out of 10 mink showed lesions ranging from one small focus of principally lymphocytes to massive lymphocytic infiltrates. In these mink bile duct proliferation or significant changes in serum gamma globulin levels were not observed at the termination of the experiment. When the euglobulin preparation was administered with the infective ex-

tract, the apparent pathogenicity of the infective material was increased. The fact that mink in Group II showed more intense signs of AD could not be related to any specific lesion absent in Group I.

Incubation of the infective extracts with peritoneal cells from infected mink did produce a disease-limiting effect as indicated by fewer deaths and only 4 out of 10 apparently infected at the termination of the experiment. Three of the 10 mink in this group showed evidence of "wasting disease" as characterized by runty appearance and splenic atrophy. One control mink which received only peritoneal cells also showed emaciation, in spite of normal food consumption, along with splenic changes. In these mink serum gamma globulin levels were normal. The gross changes in the spleens of mink which showed "wasting disease" were marked when compared to the usual

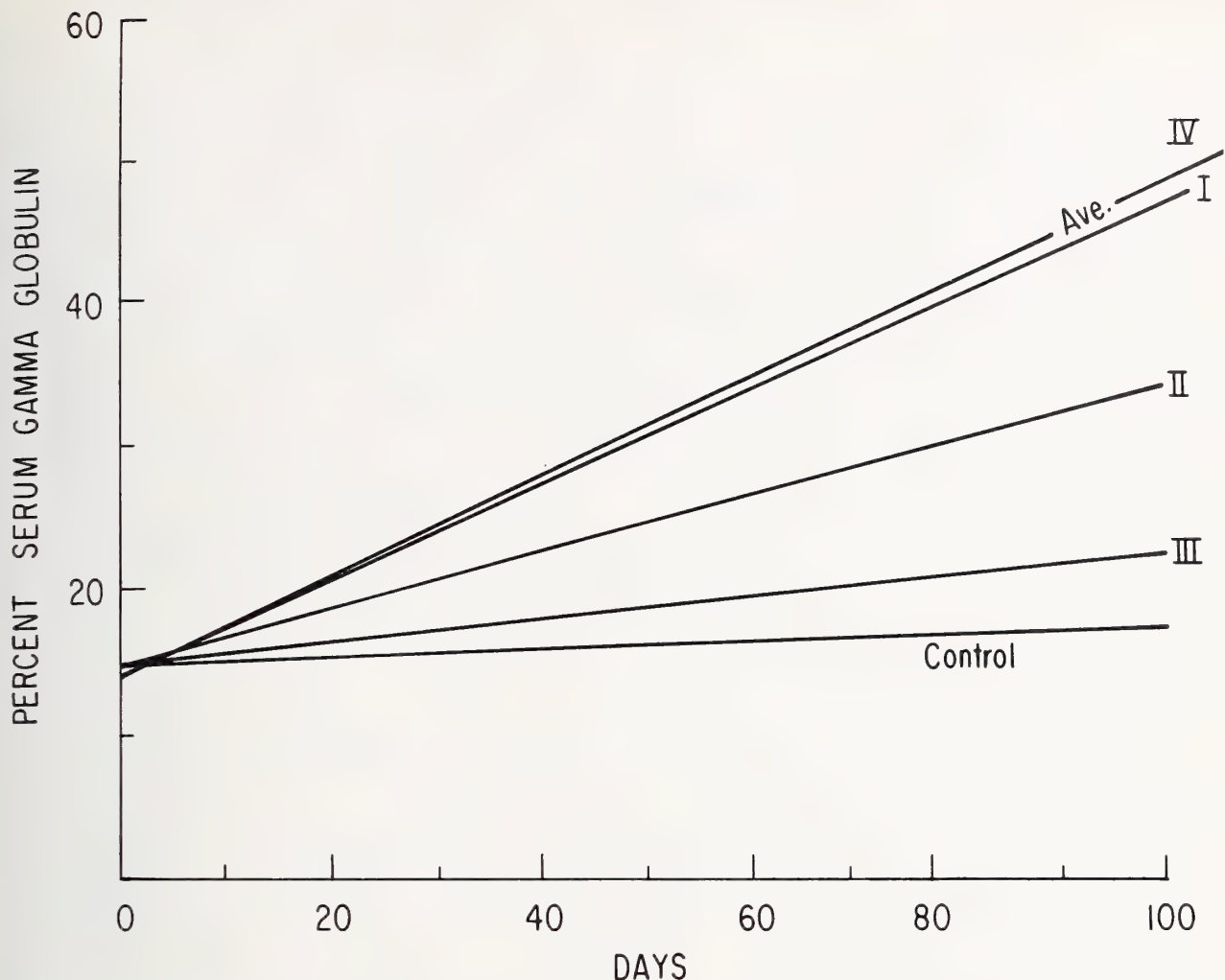


FIGURE 3.—The values plotted are the average serum gamma globulin levels of surviving mink in each experimental group.

splenic hypertrophy seen in the typical AD infected mink. The histologic difference consisted of a near depletion of lymphocytes in the white pulp of mink with "wasting disease" as opposed to increased lymphocytes and plasma cells in AD infected mink. The changes observed here may be either analogous to those described by Kaplin and Rosston (8) which were induced in F_1 hybrid mice by injecting parental strain lymphoid cells or may be representative of an altered host reaction to the AD agent.

The mink in Group IV uniformly produced a pronounced plasmacellular reaction to the infective extract-peritoneal cell suspensions when it also contained euglobulin. The response may be likened to that observed in a secondary immunization with a dramatic increase in antibody protein.

The effect of the euglobulins observed in Group II where a more apparent disease occurred and in

Group IV where a massive plasmacellular response occurred, may be indicative of a cytoimmunological reaction. If cytolytic activity was directed at virus-altered cells of the lymphoid series, and assumed to alter lysosomes as has been suggested in the absence of complement (9), the unusual cytoplasmic changes observed in leukocytes of AD infected mink and man (10, 11) may be due to autoimmunity.

Confirmation of the preliminary experiments reported herein will indicate a need for additional experimentation pertaining to the functional capabilities of the phagocytic cells in AD infected mink. The effect of AD immunoglobulins on the disease-limiting ability of leukocytes to other agents, subsequent to AD infection, will be of particular interest. In this sense, AD infection may alter the phagocytic capability of leukocytes in a manner similar to that observed in Chediak-Higashi syndrome of mink (12).

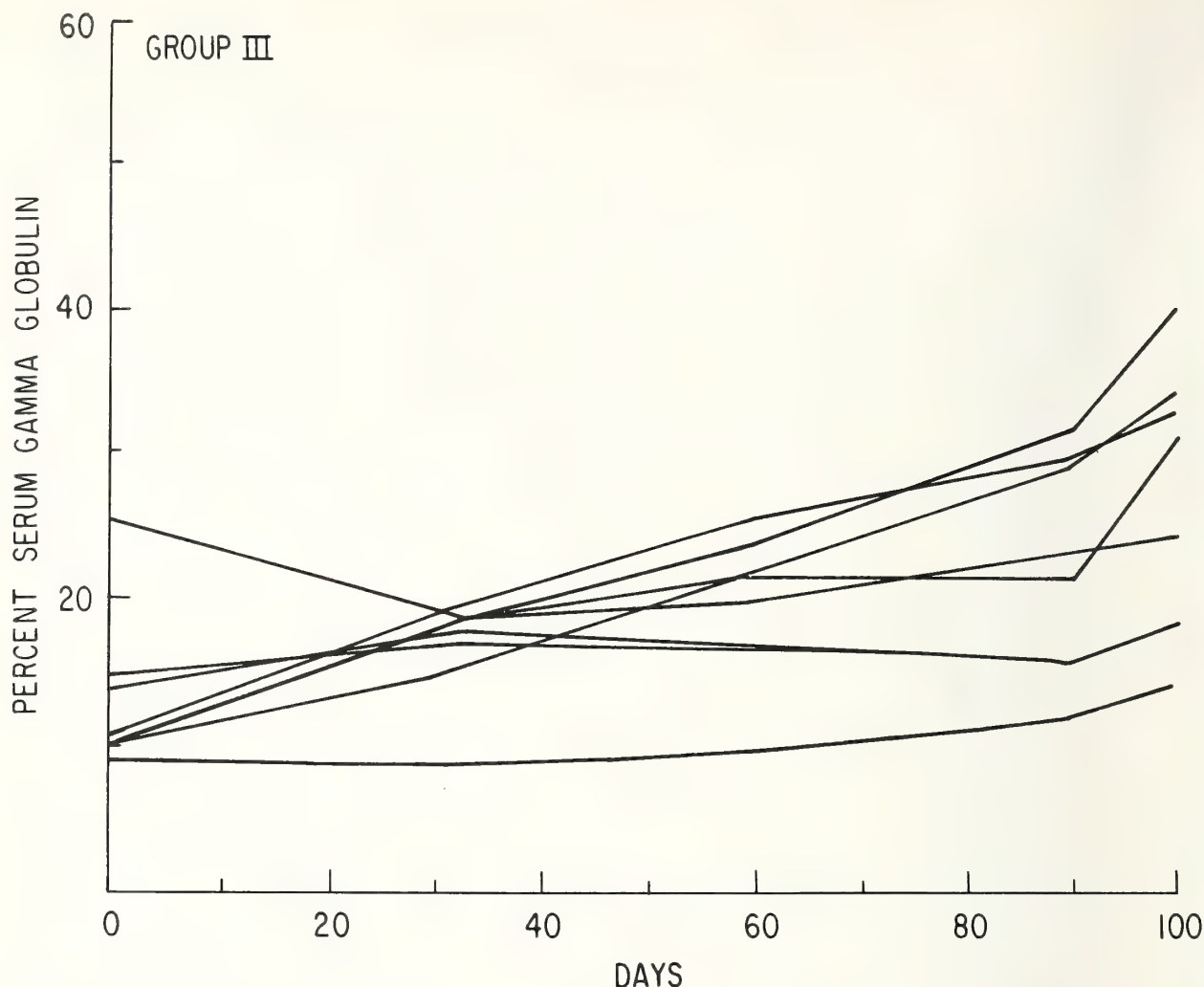


FIGURE 4.—The values plotted are percent gamma globulin from mink receiving AD tissue homogenate and “sensitized” peritoneal cells from infected mink. Death is indicated by termination of the curve at the last date of serum analysis prior to death.

SUMMARY

When “virus-free” euglobulins from mink with AD hypergammaglobulinemia were combined with infective tissue homogenates to test for disease-enhancing activity, an increased pathogenicity occurred as compared to that seen in mink which received only the infective homogenate. The disease enhancement was manifested by pronounced clinical signs and earlier mortality. In 5 of 10 control mink which received only euglobulins, some renal lymphocytic lesions were observed (serum gamma globulin levels remained normal).

When peritoneal cells from infected mink were incubated with infective extracts, infectivity was reduced to 40 percent as indicated by elevated gamma globulins and leukocytic infiltrates. Euglobulin added to a

similar infective extract-peritoneal cell suspension resulted in extensive plasmacellular infiltrates of kidney, liver, and spleen in 100 percent of inoculated mink, with gamma globulin levels near 50 percent of total serum protein. In both cases the mortality was lowered when peritoneal cells were included with the tissue extract.

ADDENDUM

During the course of Aleutian disease experimentation, it was noticed that the infected mink were more susceptible to bacterial infections than those not infected with AD. To determine whether this was specifically related to macrophage function or indirectly as macrophage function relates to antibody production, experiments were initiated which could give some estimate of the relative ability of AD infected

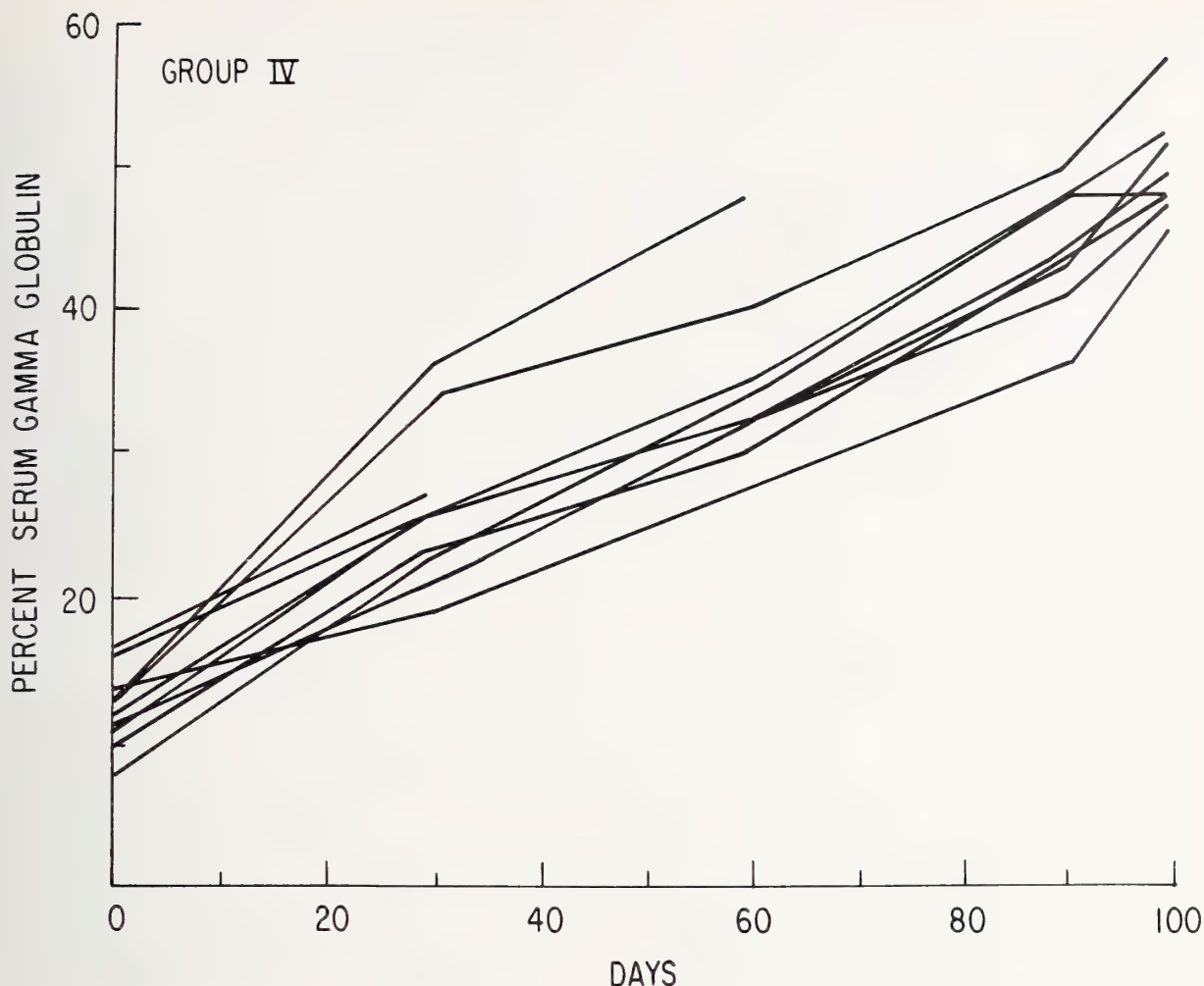


FIGURE 5.—The values plotted are percent gamma globulin of total serum protein in mink receiving AD tissue homogenate, "sensitized" peritoneal cells, and euglobulin from hypergammaglobulinemic mink. Death is indicated by termination of the curve at the last date of serum analysis prior to death.

mink to produce 19-S and 7-S antibodies. Normal mink were compared with AD infected mink in ability to form 19-S and 7-S agglutinins to a commercially prepared mixed bacterin. It was found that the average antibody production of the infected group was about one-half that of the normal with respect to 7-S immunoglobulins. However, the ability of the AD infected group to form 19-S immunoglobulins was decreased 8-fold from the average of the normal mink.

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DISCUSSION

LEADER: In regard to the type of the hypergamma-globulinemia found in Aleutian disease, do you think

that it is possible that if electrophoretic patterns on serum were run consecutively on one animal, there might be 19-S or 22-S at some earlier period which later would not appear? There is evidence in some diseases that 19-S globulin is produced early while later it changes to 7-S.

KENYON: In the particular experiments reported here we have not obtained samples before 30 days.

LEADER: Dr. Dixon pointed out that in one animal he had a change in the globulin distribution by electrophoresis from a broad heterogeneous type of electrophoretic mobility to a narrow peak at a later time in the same animal. He interpreted this as a change from a multiclonal to a monoclonal type of response.

KENYON: This has not occurred in our animals by 100 days.

Anti- γ -Globulin Factors and Immunofluorescent Studies in Aleutian Disease of Mink

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Our approach to the problems raised by Aleutian Disease (AD) was directed toward a search for various types of antibody activity in the serum of both normal and AD mink. It was of interest to try to determine whether: (1) the striking increase in γ -globulin seen in such animals constituted antibody directed towards tissue components or autologous protein in the form of anti- γ -globulin factors, and (2) if the γ -globulin fractions were localized in any particular pattern within the tissues of AD infected mink.

MATERIALS AND METHODS

Serological and Physical Studies

Sera from normal and AD mink were tested for anti- γ -globulin antibodies using various species of γ -globulins affixed to cells by tanning (1, 2) including rabbit, bovine, porcine and mink γ -globulin. Inhibition experiments were performed by incubating a dilution of agglutinating serum which produced a 2 to 3 plus agglutination with serial doubling dilutions of inhibitor starting at 1 mg./cc. After 30 minutes at 37° C. the cells tanned with the appropriate γ -globulin were added and the tests were read after standing 12 hours at 4° C. Antibody to human γ -globulin was measured by agglutination reactions of human Rh positive cells coated with human incomplete anti-Rh antibody Ripley (3). Mink sera were inactivated at 56° for one-half hour and were absorbed with one-quarter volume of packed washed sheep cells before tanned cell reaction or agglutination of human incomplete antibody-coated cells was determined. Mink aggregated γ -globulin was prepared by heating isolated normal mink γ -globulin for 10 minutes at 63° C. The molecular character of the various anti- γ -globulin factors studied was determined by sucrose density

gradient ultracentrifugation (4). Sixteen hour runs at 35,000 r.p.m. using 10 to 40 percent sucrose gradients were used for all separations.

Mink γ -globulins from both normal and AD mink were isolated by zone electrophoresis in starch (5). Quantitative precipitin curves (6) and Ouchterlony analysis followed standard procedures (7).

Immunofluorescent Studies

Fresh tissues were secured from both normal and AD infected animals and were snap-frozen in dry ice methyl cellosolve bath. No fixative was used. Sections were cut on a cryostat and stained using rabbit antiserum to mink γ -globulin conjugated with fluorescein isothiocyanate (8, 9). The rabbit antiserum to mink γ -globulin was highly specific showing strong 7-S γ -globulin and faint B₂A lines on immunoelectrophoresis against whole mink serum. The sections were examined using a fluorescent microscope and UG-1 and BG-12 filters at 90, 180 and 525 powers of magnification. Conjugated antisera were absorbed with one quarter volume of mouse liver powder before use. Specificity of staining for mink γ -globulin was established by absorbing conjugated antisera with isolated mink γ -globulin (blocking) and by staining sections with unconjugated anti-mink- γ -globulin antisera followed by staining with conjugated anti-mink- γ -globulin antisera.

RESULTS

A large proportion of sera (40 percent) from both normal and AD infected mink showed anti- γ -globulin factor activity when tested with cells tanned with various species' γ -globulins. Stronger reactions with mink γ -globulin aggregates tanned to cells than with un-

altered mink γ -globulin were notable in both normal and AD mink sera. Some slight rises in titers after AD infection were noted in many animals. The tanned cell agglutination reactions observed between mink sera and cells tanned with various heterologous species' γ -globulins could be inhibited by mink aggregated γ -globulins but not by mink γ -globulin cleared of aggregates by ultracentrifugation at 25,000 r.p.m. in the Spinco Model L ultracentrifuge for 90 minutes (table I).

TABLE I.—Agglutination and Inhibition Reactions Noted With Normal and AD Mink Sera Tested Against Cells Tanned With Various Species' γ -globulin

Animal No.	Titers with cells tanned with:				
	Mink γ -globulin	Mink γ -globulin aggregates	Rabbit γ -globulin	Bovine γ -globulin	Porcine γ -globulin
429 ¹	0	20	0	10	0
430 ¹	0	10	0	10	0
426 ²	20	160	40	20	10
619 ²	0	160	160	20	10
617 ²	20	160	160	160	40

617 agglutination of rabbit, bovine, and porcine γ -globulin tanned cells could be inhibited by mink γ -globulin aggregates but not by aggregate-free mink γ -globulin.

¹ Normal mink sera.

² AD mink sera.

In most instances the factors reacting with both mink aggregated γ -globulin tanned cells and heterologous γ -globulin tanned cells were found to reside in the 19-S fractions of sucrose density gradients. Occasional anti- γ -globulin activity in 7-S fractions was observed. The macroglobulin character of these mink anti- γ -globulin factors was confirmed in both normal and AD infected animals, and in the serum of the same animal before and after AD infection.

Concurrent immunoelectrophoretic studies of mink sera before and after infection with AD revealed that IgG and to some extent IgA (10) elevation occurred after AD infection had become established. No marked increase in IgM in AD animals was notable with the antisera used.

When normal and AD mink serum were separated into electrophoretic components by zone electrophoresis on starch, isolated mink γ -globulins were obtained. Quantitative precipitin curves using rabbit anti-mink-

γ -globulin antiserum revealed no antigenic deficiency of AD mink γ -globulin, when compared to that of normal mink. Likewise Ouchterlony analysis showed no spurs of normal mink γ -globulin over AD mink γ -globulin. Such spurs are commonly associated with antigenic deficiency seen in human M-components or myeloma proteins when similarly compared with normal human 7-S γ -globulin.

Immunofluorescent Studies

Sections of lymph nodes, kidney and liver from normal mink showed no specific staining with the conjugated anti-mink- γ -globulin antiserum; however, such tissues from AD animals showed bright fluorescence of cytoplasm of plasma cells and lymphoid cells freely dispersed throughout the lesions (fig. 1). In the livers of AD animals, fluorescence of biliary canaliculi as well as intracytoplasmic particles not definable by haematoxylin and eosin stain was noted (fig. 2). The kidney sections of 10 percent of the 15 AD animals examined showed homogeneous uniform staining of glomeruli and entire glomerular tufts; concurrent haematoxylin and eosin stains showed glomerular smudging similar to that seen with acute glomerulonephritis. Some small bright intracytoplasmic fluorescence was also noted in tubular cells of AD kidney stained with conjugated anti-mink- γ -globulin antiserum. Again the histologic localization of this bright particulate staining could not be readily assigned to any obvious cytoplasmic structure notable on haematoxylin and eosin stain of parallel sections.

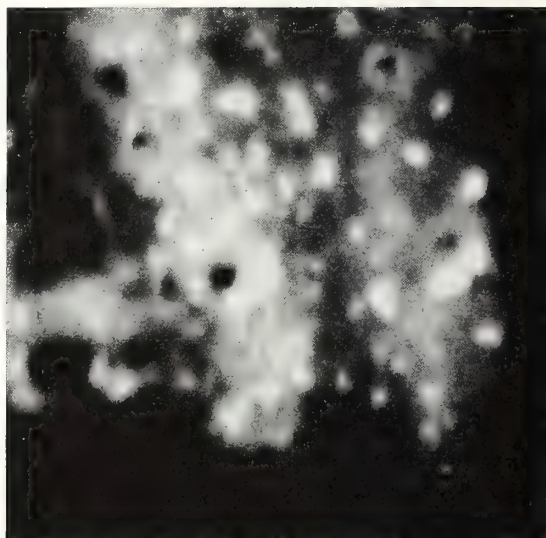


FIGURE 1.—Immunofluorescent staining of AD kidney with conjugated rabbit anti-mink- γ -globulin antiserum. Bright cytoplasmic fluorescence of plasma cells dispersed in the peritubular regions is shown. $\times 190$

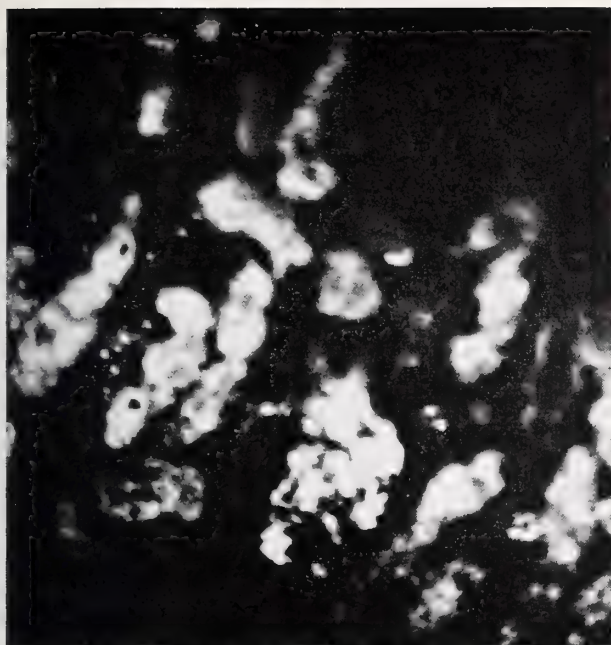


FIGURE 2.—Section of AD mink liver stained with conjugated anti-mink- γ -globulin antiserum. Bright fluorescence of the entire parenchyma of liver cells is noted in addition to plasma cells; discrete particulate cytoplasmic staining can be noted. $\times 90$

The specificity of the patterns of localization of mink γ -globulin was confirmed by standard blocking and absorption procedures.

DISCUSSION

It has already been established that most of the elevation of γ -globulin occurring in the serum of AD infected mink is IgG or 7-S γ -globulin (11). Ultra-centrifugal studies of AD mink sera performed by Porter and Dixon (12) indicated the presence of intermediate complexes in some sera. It was principally because of this latter observation that we undertook studies of the presence of anti- γ -globulin factors present in both normal and AD sera. The finding of 19-S anti- γ -globulin factors with specificity for mink γ -globulin as well as reactivity for heterologous species' γ -globulins is similar to that of human 19-S anti- γ -globulin factors or rheumatoid factors (3). The finding that the reactivity with heterologous γ -globulins could be inhibited by mink γ -globulin aggregates but not by aggregate-free mink γ -globulin suggests that the anti- γ -globulin factors present in both normal and AD sera have arisen from the antigenic stimulus of auto-logous antigen-antibody complexes; a situation similar to that observed in the various chronic infections of man also associated with rheumatoid factors might

pertain (13, 14, 15, 16). However, the presence of 19-S anti- γ -globulin factors in the mink sera is not enough to explain the overall broad elevation of γ -globulin seen in the AD sera, particularly since it is primarily elevation of IgG or 7-S γ -globulin.

The immunofluorescent studies leave little doubt that the lesions of AD in the liver, kidney and lymph nodes are replete with plasma cells making γ -globulin. The predominant pattern in all of the AD material examined was one of brightly staining plasma cells or lymphoid cells in large numbers in and around tissue lesions. The small bright particulate intracytoplasmic staining, however, remains problematical. Whether this represents the AD virus itself or localization of specific cytoplasmic antigens such as those of the lysosomes or other more complex and less well defined cytoplasmic antigens (17, 18) is not yet clear.

ACKNOWLEDGMENTS

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Virology of Visna and Other Slow and Latent Virus Infections

Chairman

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Physical, Chemical and Biological Properties of Visna Virus and Its Relationship to Other Animal Viruses

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INTRODUCTION

In the period from approximately 1935 to 1951 a number of contagious sheep diseases were observed in Iceland for the first time. Adenomatosis of the lung (1, 2), a slow progressive pneumonia called maedi (3-5) and paratuberculosis (Johne's disease) (6) could all be traced back to sheep of karakul breed which had been imported from Germany in 1933. Visna, a central nervous system disease (7), was like the above mentioned diseases unknown in Iceland before 1935 and is therefore also assumed to have been introduced by the imported sheep. The animals were, however, apparently healthy when they arrived in the country.

Visna was apparently eradicated from the field in 1951 as a result of an extensive slaughter program. Since then the disease has been maintained in the laboratory by serial transmission of diseased brain material into young healthy sheep (7, 8). Virus was later isolated in tissue culture from an infected brain. Tissue culture passages of the virus were found to cause the visna disease upon intracerebral injection (9).

In the following report our present knowledge about visna virus will be summarized. In the first section studies of various workers on the nature of the disease will be briefly described. Then studies on the physical, chemical, and biological properties of visna virus will be reviewed. In a final section the data will be discussed, the main aim being to throw a light on the relationship between visna virus and other animal viruses, particularly maedi virus, and its place in the system of animal viruses.

I. Clinical and Pathological Features of Visna

Visna was observed in the southwestern and southern part of Iceland from approximately 1935 to 1951. It occurred mostly as sporadic cases although on some farms a considerable proportion of the sheep flock showed signs of visna. The disease has been described by Sigurdsson and coworkers (7). It is usually insidious in its onset beginning with slight paresis, particularly in the hind legs. The paresis progresses slowly and usually ends in paraplegia or in total paralysis. The clinical stage of the disease can last from a few weeks to several months and seems to be invariably fatal. The clinical signs observed in transmitted cases of visna were apparently identical with the signs observed in natural cases. Transmission experiments have shown that the clinical stage of the disease is preceded by a subclinical period lasting from a few months up to several years (7, 8). During the subclinical period the main sign of the infection is increased cell count in the cerebrospinal fluid, usually beginning 1 to 2 months after intracerebral inoculation. This is accompanied by an increase in the protein content of the spinal fluid, particularly in the gamma globulins (10, 11). During this period virus may be found in the spinal fluid, whole blood, and saliva for longer or shorter periods of time and sometimes concurrently with a high concentration of neutralizing antibodies in the serum (12). Visna virus has also in numerous instances been recovered from various organs of sheep inoculated several months or even years earlier and not yet showing clinical signs. Sheep moribund from visna are consistently found to harbor the virus in various organs, and in many instances they contain

neutralizing antibodies in high titer in the serum (12, 13).

The pathology of visna has been studied by Sigurdsson and coworkers (7), Sigurdsson and Pálsson (8), Pette and coworkers (11) and Sigurdsson, Pálsson and van Bogaert (14). The primary lesion in the central nervous system is the appearance of meningeal and subependymal infiltration or proliferation of cells of the reticulo-endothelial system, preferably of lymphocytes and microglia. Perivascular infiltrations of lymphocytes are common. Demyelination of the white matter of the central nervous system seems to occur secondarily. The grey matter is usually not or only slightly affected. Pette and coworkers (11) observed hyperplasia of reticulo-endothelial elements in the lungs, lymph nodes and spleen of sheep infected with visna. No changes have been observed in other organs.

On the basis of studies on visna and the lung disease in sheep named maedi, Sigurdsson (15-17) formulated the concept of slow infections as different from acute and chronic infections. The main characteristic of slow infections, as defined by Sigurdsson, is a very long subclinical period followed by a disease running a rather regular protracted course which usually ends in death. It has been suggested that besides visna and maedi various diseases might fit into the group of slow infections, e.g., adenomatosis of sheep lungs, scrapie, mammary carcinoma of mice caused by the Bittner agent, fowl lymphomatosis, Rous sarcoma, and mouse leukemia (15-17).

II. Physical, Chemical, and Biological Properties of Visna Virus

Growth in Tissue Cultures.—Visna virus propagates readily in tissue cultures from various organs of sheep, both in primary cultures and in serially propagated cells. Cultures of the latter type, derived from the choroid plexus of sheep brain, have been used in almost all studies with the virus. The propagation of visna virus in cell cultures is accompanied by cytopathic changes consisting mainly in the formation of multinuclear giant cells which in unstained cultures appear as large stellate cells with increased refractility. In most instances the infection leads to a complete disintegration of the cultures.

Visna virus propagation accompanied by cytopathic changes occurs in cultures of kidney cells and plexus cells from calves. In plexus cultures from human embryos and from pigs, guinea pigs, dogs and cats visna virus persisted in low titer for a period of 1 to 3 months

and a few multinuclear giant cells were observed in the cultures at the end of the period. No viral propagation has been observed in cultures of human amniotic cells, chick embryo fibroblasts, HeLa cells and L cells (9, 18, 19).

Growth Cycle.—In monolayer cultures of sheep cells inoculated with an input multiplicity of 10 to 20 TCID₅₀ of visna virus per cell the latent period is 20 to 22 hours. This is followed by a period of rapid virus multiplication lasting for about 1 day after which the infectivity titer levels off at about 10⁷ to 10⁸ TCID₅₀ per ml. Virus multiplication is accompanied by a cytopathic effect which in the course of a few days leads to detachment of the cells from the glass.

During the whole period of virus multiplication the amount of cell associated virus (CAV) is equal to or less than the amount of released virus in the fluid. Most of the CAV is apparently located on the external surface of the cells (19-21).

Mode of Replication in Host Cells.—Electron microscope studies have shown that the virus particles are formed by budding at the external membrane of the host cells. They seem to be released as two-walled bodies which later contract to form the final virus particles outside the cells. Virus particles have not been observed within the cells. This is in accordance with studies of the growth cycle which indicate that infectious virus is formed at the cell membrane at the time of release and is not accumulated within the cells (20, 21).

Size and Structure.—The size of the visna particles varies considerably when studied by the electron microscope. In osmium fixed preparations embedded in methacrylate most of the particles vary between 70 and 100 m μ in diameter with an average of 85 m μ . The particles are roughly spherical and contain a centrally located osmiophilic core separated from the outer membrane by a zone of low electrone density. The size of the central core varies from 30 to 40 m μ with an average of 35 m μ .

When studied by the negative staining technique the particles appear somewhat bigger than in preparations embedded in methacrylate, most of them having a diameter around 90 to 100 m μ . The surface of the particles is covered with numerous projections about 100 Å in length. No internal structure could be made out with certainty with this technique although concentric ring structure has been observed in the interior of some particles (20, 22).

Nucleic Acids.—The nucleic acid composition of the visna virion is unknown. Experiments with 5-bromodeoxyuridine and actinomycin D indicate that

formation of the virus depends on synthesis and function of DNA. The DNA synthesis is initiated shortly after infection and completed in a few hours, preceding virus formation by 15 to 20 hours. DNA-mediated RNA synthesis, on the other hand, seems to occur shortly before the release of infectious virus (19, 23).

Sensitivity to Chemical Agents.—Visna virus is inactivated by incubation with ethyl ether at 4° C. for 24 hours and by shaking with ether at 21° C. for 10 minutes. This treatment causes a complete disintegration of the virus particles. The virus is inactivated by metaperiodate, chloroform, ethanol, and phenol. Incubation with 0.04 percent formaldehyde at 19° to 21° C. inactivates the virus rapidly in the beginning, then more slowly causing loss in titer of more than 6 log₁₀ units in less than 2 days. Incubation with 0.05 percent trypsin at 36° to 37° C. for 30 minutes inactivated visna virus by more than 5 log₁₀ units. The effect of trypsin was abolished in the presence of soybean trypsin inhibitor (19, 22, 24).

Sensitivity to Physical and Physiochemical Agents.—Visna virus is stable to sonic treatment (800,000 cycles per second) for at least 30 minutes and to at least three cycles of freezing and thawing. It is inactivated by UV light with maximum emission at 2537 Å. The inactivation rate is exponential and is about 10 times slower than that of herpes virus, poliovirus, and NDV. Visna virus is inactivated by visible light in the presence of toluidine blue at the same rate as vaccinia virus. The virus is completely inactivated by heating to 56° C. for 10 minutes. The 90 percent inactivation rate in medium 199 with 1 percent sheep serum is 10 to 15 minutes at 50° C., 24 to 30 hours at 37° C., about 9 days at 19.5° C., and about 4 months at 4° C. The inactivation rate is more rapid in medium without serum. The virus is stable for months when stored at -50° to -60° C. Visna virus is relatively stable in the range of pH 5.1 to 10, but it is most stable at slightly alkaline pH. At pH 4.2 or lower the virus is rapidly inactivated, losing more than 4 log₁₀ of its infectivity in 30 minutes at pH 3.2 (19° to 21° C.) (19, 21, 24-26).

Hemagglutination and Hemadsorption.—Visna virus does not agglutinate red cells from sheep, calf, horse, dog, cat, guinea pig, rabbit, rat, mouse, hamster, and chick or human type 0 red cells when these are incubated with virus in 0.01 M phosphate buffered saline, pH 7.1, at various temperatures. There is no hemadsorption of any of these various types of red cells by cell layers infected with visna virus (19).

Serological Tests.—Neutralization test is the only serological test so far available for visna virus. It has

been standardized and found to be a reliable method for assay of neutralizing antibodies in sheep sera. It is necessary to preincubate virus-antiserum mixtures for a considerable length of time before inoculation into tissue cultures in order to obtain maximal neutralization of virus. The incubation time depends on the temperature. Omission of preincubation results in neutralizing titers about 50 to 100 times lower than those obtained after preincubation at 37° C. for 3 hours. The neutralization line determined under standard conditions was found to have a slope of about 4.0. A 10-fold increase in virus concentration therefore causes a 1.8-fold decrease in neutralizing serum titer (25, 27).

Antigenic Relationships.—All strains of visna virus which have been compared by neutralization test are antigenically related, although apparently not identical. They are all neutralized by antisera against maedi virus and also by sera from natural cases of maedi. Human sera have been found to neutralize visna virus in low titer. This, however, seems to be due to nonspecific inhibitors rather than to antibodies so that a serological relationship with a human virus, or viruses, is not indicated. Visna virus is neutralized by bovine sera, often in high titer, but it is still unknown whether this is due to nonspecific inhibitors or to a serological cross reaction with a bovine virus. Antisera against Rous sarcoma virus, mouse leukemia viruses of Gross, Friend and Moloney, measles virus, mumps virus and influenza A virus were all negative in neutralization test against visna virus (18, 27, 28).

III. The Relationship between Visna and Maedi Viruses and Their Affinity with Avian and Murine Leukemia Viruses

The main characteristics of visna and maedi viruses are summarized in table I. A comparison of the data shows a close relationship between the two viruses with respect to physical, chemical and biological properties, including serological reaction. A quantitative difference is noted (1) in their growth rate in monolayer cultures of sheep cells, (2) in their inactivation rate at low pH, and (3) in their sensitivity to neutralization by visna antisera. It is not known to what degree these differences are due to the long history of laboratory transmission of visna prior to the present study of visna virus. At any rate, the differences between visna and maedi viruses are not greater than those frequently found between various strains or types of a virus (29-32).

TABLE I.—A Comparison of Some Properties of Visna and Maedi Viruses and the Viruses of the Avian Leukosis Complex (19, 27, 33–57)

Property	Visna	Maedi	Avian leukosis viruses
Size and structure of particles embedded in methacrylate	Particles: 70–100 m μ Central core: 30–40 m μ	Particles: 60–90 m μ Central core: 30–40 m μ (33, 19).	Particles: 70–80 m μ (34) Central core: 30–40 m μ
Size and structure of particles in PTA	Particles, 90–100 m μ covered with spikes (100 Å)	Not studied	Particles, 90–110 m μ covered with spikes (35, 36, 37)
Site of virus maturation	Cell membrane	Cell membrane (19)	Cell membrane (38, 39, 40)
Growth curve	Latent period	About 30 hours	12 hours (RSV), 24 hours (AMV)
	Period of increase in virus titer	2–3 days (19)	1 day (RSV), 2–3 days (AMV) (41, 42)
CPE in tissue culture	+	+ (33)	(+) (RSV) (43, 44)
Transformation of cells in tissue culture.	Not observed	Not observed	+ (45, 46)
Nucleic acid composition	Unknown	Unknown	RNA (47, 36)
Requirement for DNA synthesis	+ Early in the latent period	+ (19)	+ (RSV) Shortly after exposure to RSV (49, 50)
Sensitivity to actinomycin D	+ During virus formation	Not studied	+ (RSV) During virus formation (49, 50, 51)
Sensitivity to UV irradiation	10 times more resistant than NDV	10 times more resistant than NDV (19)	10 times more resistant than NDV (52, 53)
Sensitivity to ether and chloroform	+	+ (33, 19)	+ (53, 54)
Time of 90 percent inactivation at 50° C.	10–15 minutes	10–15 minutes (19)	45 minutes (55)
Sensitivity to low pH	90 percent inactivation 1½ hr. at pH 4.2	90 percent inactivation one-half hr. at pH 4.2 (19)	Labile at low pH (56)
Hemagglutination	Not observed	Not observed (19)	Not observed
Serological relationship	Neutralized by maedi antisera	Partly neutralized by visna antisera (27)	Antigenically interrelated (57)
	Not neutralized by RSV antiserum (27)		

Concerning the relationship to other known animal viruses, the morphology of the virus particles and some of their physical, chemical, and biological characters remind one of avian and murine leukemia viruses. A comparison with the viruses of the avian leukosis complex is included in table I. There are striking similarities, the most noticeable ones being the long latent period, the requirement for DNA synthesis shortly after infection, and the relatively great resistance to UV light irradiation. On the basis of physical, chemical, and biological characters visna and maedi

viruses can therefore be tentatively placed close to the groups of avian and murine leukemia viruses in the animal virus system (58). Visna and maedi viruses are much more cytopathic in cell culture than are the leukemia viruses, but do not cause transformation of cells, as far as we know. Under certain conditions, however, visna virus shows a very moderate activity in tissue cultures and can persist in the same culture for months without causing much CPE (9, 18). The fate of visna and maedi viruses in infected animals also reminds one of the leukemia viruses (59), at least in

certain respects. Thus, virus multiplication continues in the infected animals for a long time before clinical signs appear, and despite the presence of neutralizing serum antibodies which do not protect the animals against illness (12, 13, 60).

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DISCUSSION

GAJDUSEK: First of all, I would like to congratulate Dr. Thormar on the first thorough virology completed in the field of slow viruses of the nervous system. Are you finding any complement-fixing antibody to visna?

THORMAR: We have not been able to demonstrate complement-fixing antibody.

GAJDUSEK: The infectivity titers are high enough for it, don't you agree?

THORMAR: Yes.

GAJDUSEK: How soon after infection is neutralizing antibody detectable?

THORMAR: This varies. Usually a few months.

GAJDUSEK: Are you still isolating virus at this time?

THORMAR: Yes, we are still isolating virus.

BANG: I would like to ask if there is any evidence that virus spreads in the presence of antibody, in view of the fact that you are finding positive sera with high antibody titer.

THORMAR: We have not so far tried to demonstrate this.

Chronic Disease Following Lymphocytic Choriomeningitis Virus Inoculation and Possible Mechanisms of Slow Virus Pathogenesis¹

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Lymphocytic choriomeningitis (LCM) infection of mice has long been known to occur in two forms; the first, an acute usually fatal disease characterized particularly by meningitis but also including hepatitis, pneumonitis, etc. (1, 2), and the second an inapparent or latent form which is continuously transmissible through many generations (3, 4). A long-lasting inapparent infection was also shown to result from inoculation of newborn mice with LCM virus (4). Study of the induction of this condition (5) indicated that it depended upon the ability of the host mice to develop immunological tolerance to an intrinsically harmless strain of LCM virus; the long-lasting infection was therefore called persistent tolerant infection (PTI). Infected newborn mice frequently passed through a temporary "runt" condition lasting for 3 to 4 weeks after which full recovery occurred with rapid gain in weight and appearance comparable to control uninoculated animals. Further study (6) showed that ability to cause tolerance was associated with intraperitoneally passed "viscerotropic" virus strains harvested from the liver. Such tolerance inducing strains were termed "docile" by comparison with "aggressive" intracerebrally passed strains which caused a lethal response in newborn mice. This response was similar to LCM disease of adult mice caused by both strains; it appeared to be due to an immunological conflict which was evidently responsible for acute death in adult mice after infection with LCM (5, 7).

The healthy appearance of young LCM-PTI mice and their general indistinguishability from normal controls gave the impression that the immunological tolerance to the viral antigen was complete, and would remain so for the normal life span of the animal. Previous attempts to break down the tolerance failed (4) and although a few animals were kept, and shown to remain tolerant at least as long as 195 days, with virus titers of 10^6 – 10^7 LD₅₀/gm. in the brain and of 10^4 – 10^5 LD₅₀/ml. in the blood, no long-term study of PTI mice was made. Occasional observations of a few PTI animals saved for possible future use indicated that these animals did not remain as healthy as controls. The following studies of the available stock of relatively old PTI animals were then made in an effort to assess the long-term outcome of tolerant LCM virus infection, the preliminary observations of which indicated that the virus was responsible (8) for a late onset or slow disease process.

MATERIALS AND METHODS

Virus Strains.—Most of the experiments were done with a strain (No. 57135) of LCM virus, referred to as UBC, which was the same as that used in previous work and was originally derived from the W. E. strain, Rockefeller Institute. The tolerance inducing capacity of this strain and its derivatives has been described previously (6); the substrains were named according to their passage history, which determines their docility or tolerance inducing capacity; thus the M/B₁ strain had one mouse brain passage since receipt of the original (guinea pig passed) material and was relatively docile; M/B₇ had received 7 brain passages and was

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highly aggressive (nondocile); M/B₆L₁₁ had received 6 brain passages and 11 liver passages and was very docile. M/B₂E₃₅ was an egg-adapted docile substrain of the first brain passage of a different strain of LCM, No. 4707, designated the Albany strain, which had been isolated from a human infection. All stock virus was tested and found to be bacteriologically sterile with the exception of one batch of M/B₆L₁₁ which was contaminated with a Gram-negative bacterium. The organism was apparently harmless and the batch was used in one experiment but at a dilution at which the contaminant was diluted out; subsequently a new bacteriologically sterile batch was used.

Virus Stock Pools.—Twenty percent organ suspensions in GTH diluent (gelatin 0.05 percent in Hanks' saline buffered with tris in place of bicarbonate) were homogenized in an omnimix. After clarifying by centrifugation, stocks were quick-frozen with dry ice alcohol in screw-capped vials and stored at -80° C. The pools showed no significant variation in titer whether titrated immediately after freezing or after intervals of a few months. Normal mouse brain pool was prepared as above from the second "blind" pass of uninoculated mouse brain.

Mice.—Albany strain albino mice were used throughout; stock animals were maintained in isolation in a closed colony developed 22 years ago from a local source of albino mice.

Eperythrozoon coccoides.—The strain of *Eperythrozoon coccoides* was the same as that used previously (9) and was passaged weekly in normal Albany mice by inoculation of a 10^{-6} dilution of heart blood of infected mice.

Bleeding.—Routinely, orbital bleeding of the mice under ether anesthesia was performed using a Pasteur pipette; occasionally heart bleeding was used on animals to be sacrificed. When whole blood was required, it was collected into a vessel containing dried oxalate; for virus titration, blood was diluted quantitatively into heparinized GTH. Red cell counts were made by standard diluting and hemocytometer counting procedures using fresh oxalated or heparinized blood and Gower's solution as diluent.

Immune Mice.—To obtain LCM immune mice, young adult 10 to 12 gm. Albany mice were injected 3 times with a 10^{-8} dilution of M/B₇ virus at 12-day intervals, by subcutaneous (sc), then intraperitoneal (ip), then intracerebral (ic) routes. The mice were generally used 2 to 4 weeks after the last injection at which time their serum had a complement-fixation titer, against LCM virus antigen, of 40 to 50 (reciprocal of extrapolated 50 percent endpoint dilution).

Immune Cells.—Cells were prepared from the spleen or thymus by the sieve method described by Billingham and Brent (10). The cells were washed twice by centrifugation in GTH and finally suspended in GTH for injection; they were enumerated by means of a hemocytometer.

Pathological Studies.—No detailed pathological studies were made. The histological examinations reported here were kindly made on a routine basis by Dr. D. N. Collins of this laboratory on sections stained with Harris' hematoxylin and eosin from tissues removed at autopsy and placed in Bouin's solution.

Parabiosis Operations.—Mice were clipped on the appropriate sides using a close-cutting electric clipper fitted with the suction tube of a vacuum cleaner to remove infected hair clippings. An operative technic developed from that described by Martinez, Smith, Shapiro and Good (11) was used. After anesthetization by ip injection of 0.01 ml./gm. nembutal solution (containing chloroform as a preservative) occasionally with the addition of a little ether, lateral skin incisions approximately $1\frac{1}{2}$ inches long were made on each mouse and the skin freed for three-eighths inch around the incision. Matching one-half inch to three-fourths inch-long incisions were then made longitudinally through the muscle and peritoneum of the lateral aspects of the body walls of the two mice from just below the diaphragm to above the iliac crest. The peritoneal cavities were then joined by a series of continuous silk sutures placed so as to evert and oppose the peritoneal surfaces. Skin was closed with closely placed skin clips and the mice kept warm until fully conscious. All blood vessels were avoided.

Neonatal Cell Inoculation.—To minimize cross immunological reactions between parabiotic mice, groups of animals intended for parabiosis were rendered mutually tolerant by inoculation with cell suspensions at birth by the following procedure. Immunological differences were thought unlikely to be significant since the mice used were from a small closed colony which had been maintained in isolation over a period of 22 years, and tests showed that these mice accepted skin grafts from each other with survival for at least several months. Mice for the parabiotic studies were prepared in batches of 15 litters, obtained 5 to 18 hours after birth; 1 mouse was removed from each litter, and the spleen and thymus of each were removed and pooled; the cells were extracted from these (as described above) and injected intravenously (iv) into each of the remaining baby mice in all the litters, so that each mouse received 1.2×10^4 cells.

RESULTS

The Long-Term Effects of Persistent Tolerant Infection of Mice With LCM Virus.—The survival of mice following inoculation with a docile strain of LCM virus (M/B₆L₁₁) was measured for a 7 month period, and compared with controls inoculated with normal mouse brain. A 10⁻³ dilution was employed in each case, giving a virus dosage of 6 × 10³ LD₅₀ (for adult mice) per 0.03 ml., the inoculum size. Sufficient litters of mice were used to give approximately 100 infant mice which were all inoculated between 24 and 48 hours after birth. Litters were kept in individual cages throughout the experiment. The number of mice which survived during the period of the experiment is shown in figure 1. The 2 curves were almost horizontal after the first 4 weeks and were almost identical; however, the virus infected mice had a very slightly higher rate of death. Both groups of animals suffered almost 20 percent mortality during the first week, due to inoculation deaths and maternal failures; during the next 3 weeks the virus infected group showed a few deaths and a fairly high incidence of runs (fig. 2A); the control mice at this time appeared perfectly well (fig. 2B) and weighed twice as much as the runs. The runt phase proved to be transitory, followed by rapid recovery by the fifth week when they were indistinguishable from controls, but weighed less (fig. 2 C and D). The changes in average weight of the mice in the two groups are shown in figure 3; after the first 3 weeks during which runs occurred, the average weight in the virus infected group rose rapidly and, though gaining on the controls for 2 weeks, failed to make up the gap which was 3.8 gm.

at the closest point (10 weeks), thereafter slowly increasing to reach 6 gm. by the 24th week. In spite of their 19 percent lower weight, the virus infected group appeared to be perfectly healthy; random mice were tested for LCM viremia during the experiment and all proved positive. The conclusion was made that some impairment of weight gain was the only abnormality present in long-term PTI mice, apart from the persistent viremia previously reported; at this time the experiment was terminated owing to pressure for space. This was unfortunate since later it was noticed that other small stocks of older PTI mice were showing occasional sick mice, and it appeared that some type of disease was occurring in a random fashion in occasional mice. These stocks represented residual mice from experiments concerning the induction of tolerance and were retained to provide a pool of PTI mice for use in other experiments. Records of PTI mouse stocks showing totals of surviving animals were examined after making corrections to allow for animals removed for experiments, and are shown as survival curves in figure 4. These curves show a fairly high rate of mortality (resulting both from deaths in the cage and from mice removed and killed because of marked sickness or severe biting). The M/B₇, an aggressive strain which does not readily induce tolerance, gave the highest rate of mortality (curve B); both the M/B₁ strain (curve C), which is relatively docile and the very docile M/B₆L₁₀ strain (curve A) showed a lower mortality rate which was comparable with the mortality rate shown by control uninoculated mice (curve D) up to about 12 months; thereafter the PTI mice had a higher death rate than the controls. It

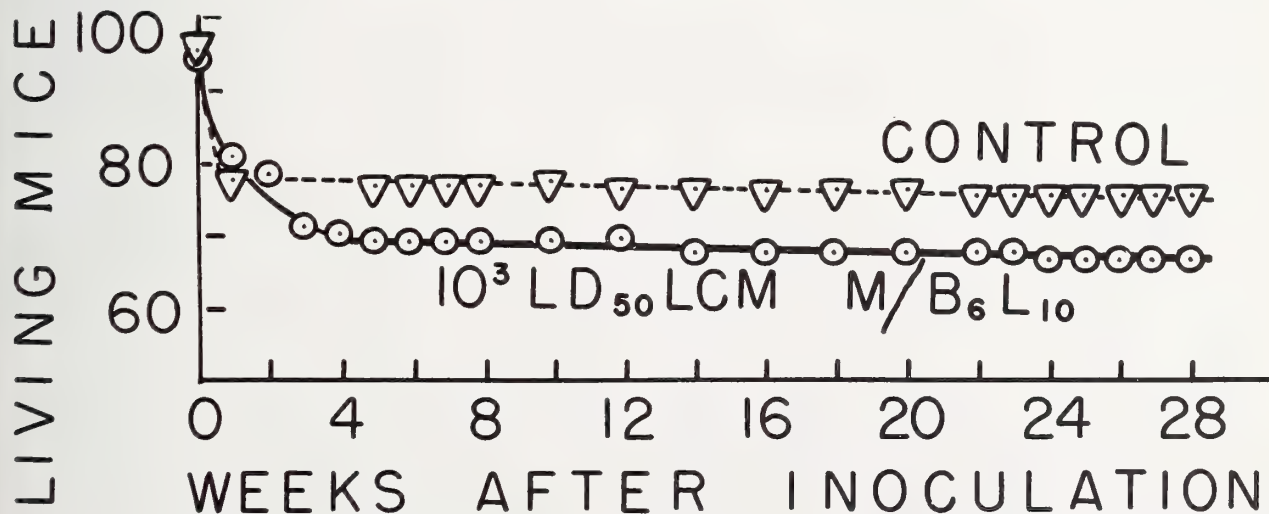


FIGURE 1.—Curves showing the number of mice surviving ip inoculation with 10³ LD₅₀ LCM virus (solid line). Control mice inoculated with normal mouse brain suspension shown by a broken line.

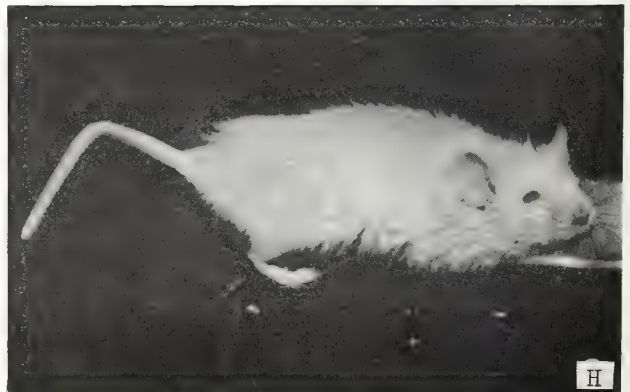
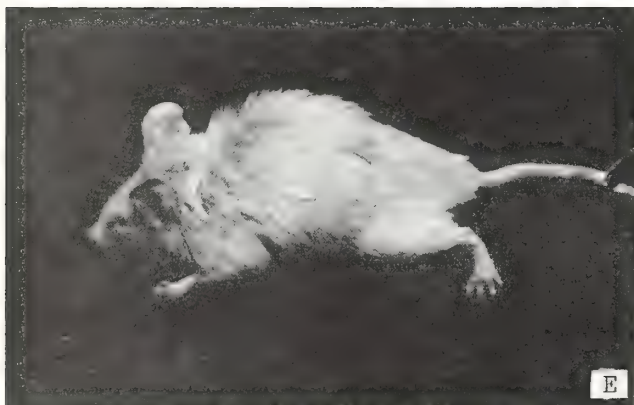
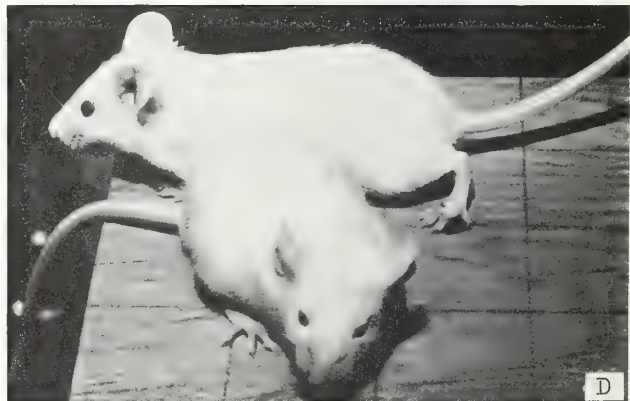
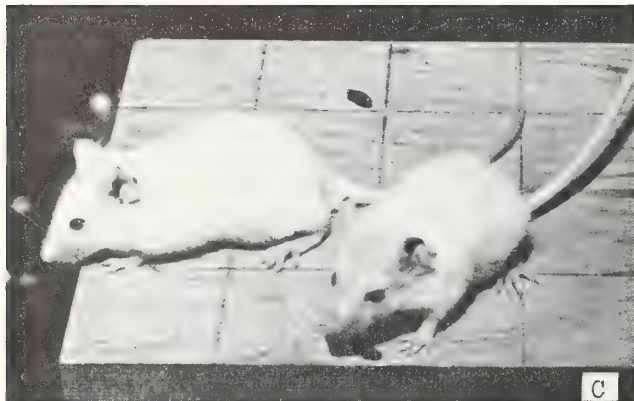


FIGURE 2.—A. Two PTI mice showing marked runting, photographed 4 weeks after ip inoculation with virus (at birth). Weights: 5.9 and 6.3 gm. B. Two normal control mice, photographed 4 weeks after ip inoculation with normal mouse brain suspension (at birth). Weights: 12.9 and 14.2 gm. C. Same PTI mice as in A, photographed 1 week later (5 weeks after virus inoculation). Weights: 10.5 and 11.5 gm. D. Same normal mice as in B, photographed 1 week later (5 weeks after inoculation). Weights: 16.5 and 18.5 gm. E, F, G. Three PTI mice showing late disease 15 months after birth and inoculation ic with M/B₁ LCM virus. H. PTI mouse showing late disease 21 months after birth and inoculation ic with M/B₁ LCM virus. A, B, C, D and E reproduced by permission from Cold Spring Harbor Symp., 27, 479, 1962 (8).

appeared likely that the greater rate of mortality shown by both PTI and control mice in these results, compared with the first experiment, may have been due to stress imposed on the mice by the crowded conditions in the large cages used for these mouse pools.

In order to ascertain the titer of virus carried in the mice after an interval of 13 and 20 months (the

maximum ages of PTI mice at that time), random animals were selected and sacrificed. The results (8) of titrations of suspensions of the brain, liver and blood of these animals showed virus titers of 10^4 – 10^6 LD₅₀ in all cases, except a single M/B₇ mouse in which brain and liver only showed 10^2 LD₅₀; in general, liver titers were about one log₁₀ unit higher than brain or blood. The fact that these titers were within 2 log₁₀ units of the maximum titers found in PTI mice shortly after inoculation, or in adult mice 2 days following ic inoculation (7), confirmed that they were sufficiently tolerant to allow very high levels of virus multiplication to persist. At this time sick animals occurring in the PTI stock pools were no longer killed but were saved; it was found that the sick animals first showed ruffled fur, and frequently blepharitis and a hunched posture, a combination somewhat similar to the early stages of acute LCM disease. However, in the advanced PTI animals, the resemblance disappeared; they showed no tendency to convulse, but developed degenerative changes in their eyes and in their skin,

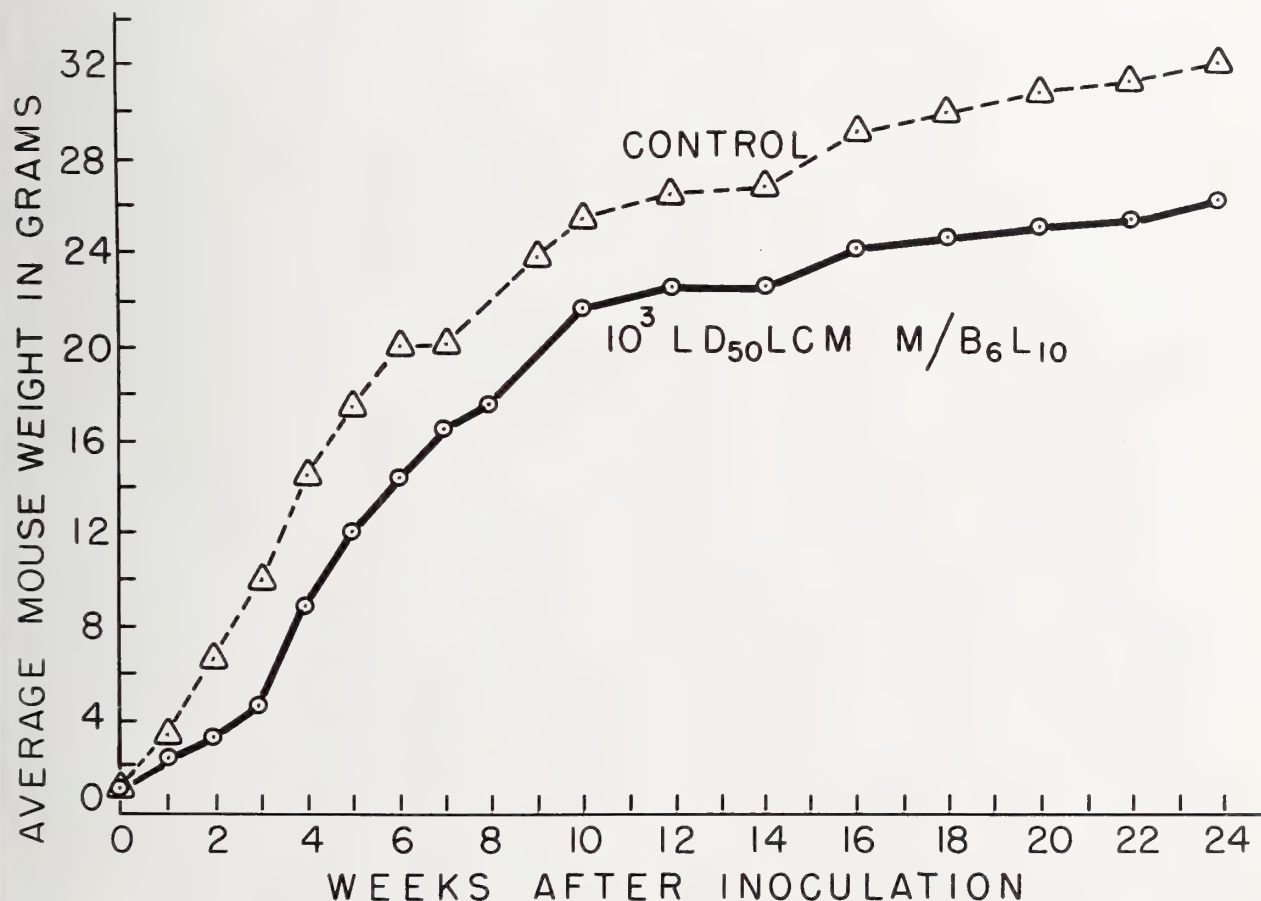


FIGURE 3.—Weight curves of control (broken line) and virus-infected PTI mice (solid line). Same experiment as figure 1 and figure 2 A–D. Reproduced by permission from Cold Spring Harbor Symp., 27, 479, 1962 (8).

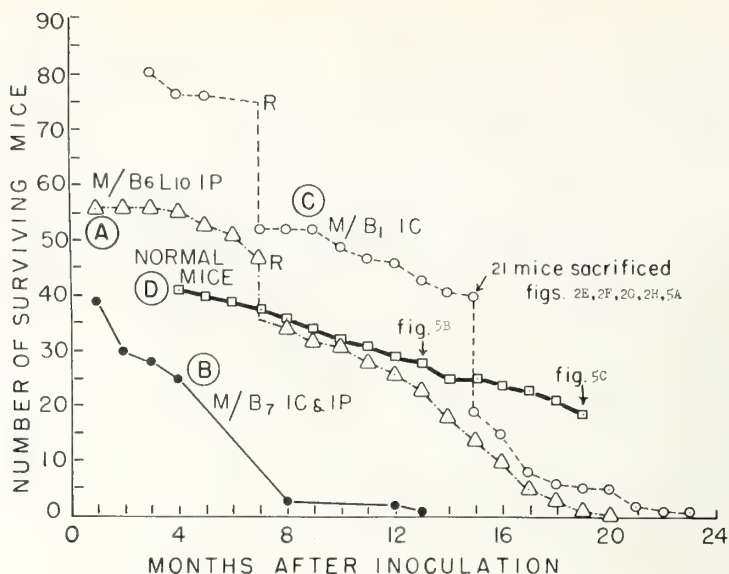


FIGURE 4.—Survival curves for 3 stocks (A, B, C) of PTI mice kept in large cages. An unknown number of mice were removed at R on curves A and C. Curve D shows the survival of normal, uninoculated control mice under the same experimental conditions. Figure numbers refer to photographs of representative mice.

with loss of hair and a dilapidated “moth-eaten” appearance. The tail was thinner than normal and was relatively stiff, frequently maintaining a constant shape instead of bending freely as the animal moved about. Movements of the limbs appeared stiff, but no obvious arthritis could be detected. This disease pattern was referred to as “late disease.” The appearance of PTI mice with late disease due to M/B₁ LCM virus 15 and 21 months after neonatal ic inoculation is shown in figure 2 E to H and figure 5A. The extremely dilapidated appearance of these sick PTI animals was in marked contrast to the sleek fur and healthy condition of the uninoculated mice (figure 5 B and C) 13 and 19 months after birth. No “late disease” was seen in any control animals. Six PTI animals showing the above signs of disease were sacrificed and subjected to postmortem examination. No clear pattern of morbid change was evident by naked eye, except that all the animals had smaller spleens than expected. On histological examination after fixation in Bouin’s solution, no abnormalities were noted in the brain, but congestion and occasional foci of small mononuclear (? lymphocytic) cells were present in the liver, and multiple focal infiltrations of similar cells were present in the kidneys. One mouse (7 months after inoculation) was found to have a lymphosarcoma or reticulum cell sarcoma filling the thoracic cavity, with infiltration of the lungs, heart and mediastinum,

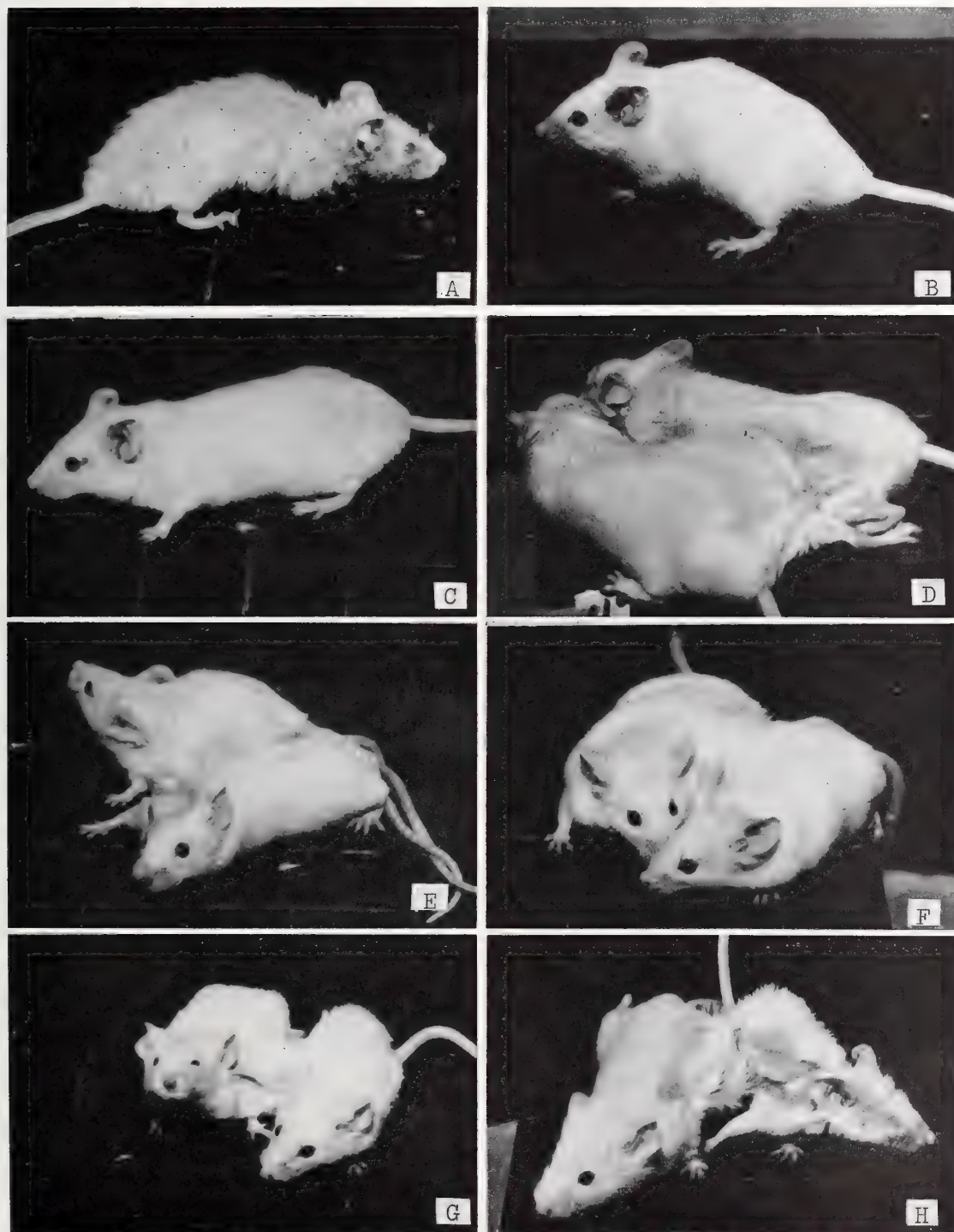
intercostal muscles and bone marrow of the ribs; the splenic tissue was similarly infiltrated. In another, there was considerable splenic pigment deposition believed to be hemosiderin, hepatitis, and suppurative pyelonephritis with abscess formation; a second mouse also showed suppurative pyelonephritis and abscess formation and in this case the spleen was moderately hyperplastic.

The Effect of *Eperythrozoon coccoides* Upon PTI Mice.—It seemed likely that a disturbance in the equilibrium between the multiplication of LCM virus and the tolerance of the host was responsible for the

FIGURE 5.—A. PTI mouse showing late disease 21 months after birth and inoculation ic with M/B₁ LCM virus. B. Normal uninoculated mouse 13 months after birth. C. Normal uninoculated mouse 19 months after birth. D, E. Two different pairs of normal/normal parabioc mice showing normal appearance 120 days (4 months) after joining. F. Normal/immune parabioc mice showing normal appearance 195 days (6½ months) after joining. G. PTI/immune parabioc mice 120 days (4 months) after joining. This was the least sick pair; the PTI animal on the right showed only slight ruffling of fur and some wasting. All PTI mice were joined to the left side of the immune. H. PTI/immune parabioc mice 90 days (3 months) after joining. The PTI animal showed severe wasting, ruffled fur, alopecia, ophthalmitis, and anemia. A, C, F and H reproduced by permission from Cold Spring Harbor Symp., 27, 479, 1962 (8).

onset of disease in the older PTI animals. Attempts were therefore made to upset this equilibrium in the hope of breaking down the immunological tolerance of the host using superinfection of PTI mice with *E. coccoides*. This agent has been shown (9) to convert a subclinical infection due to sc or ip injection

of LCM virus into a severe disease with about 70 per cent mortality some 16 days later. It seemed likely that this agent might similarly induce acute disease in PTI mice if given to them some weeks or months after they had acquired their tolerant infection. An experiment to test this was therefore undertaken, using two PTI



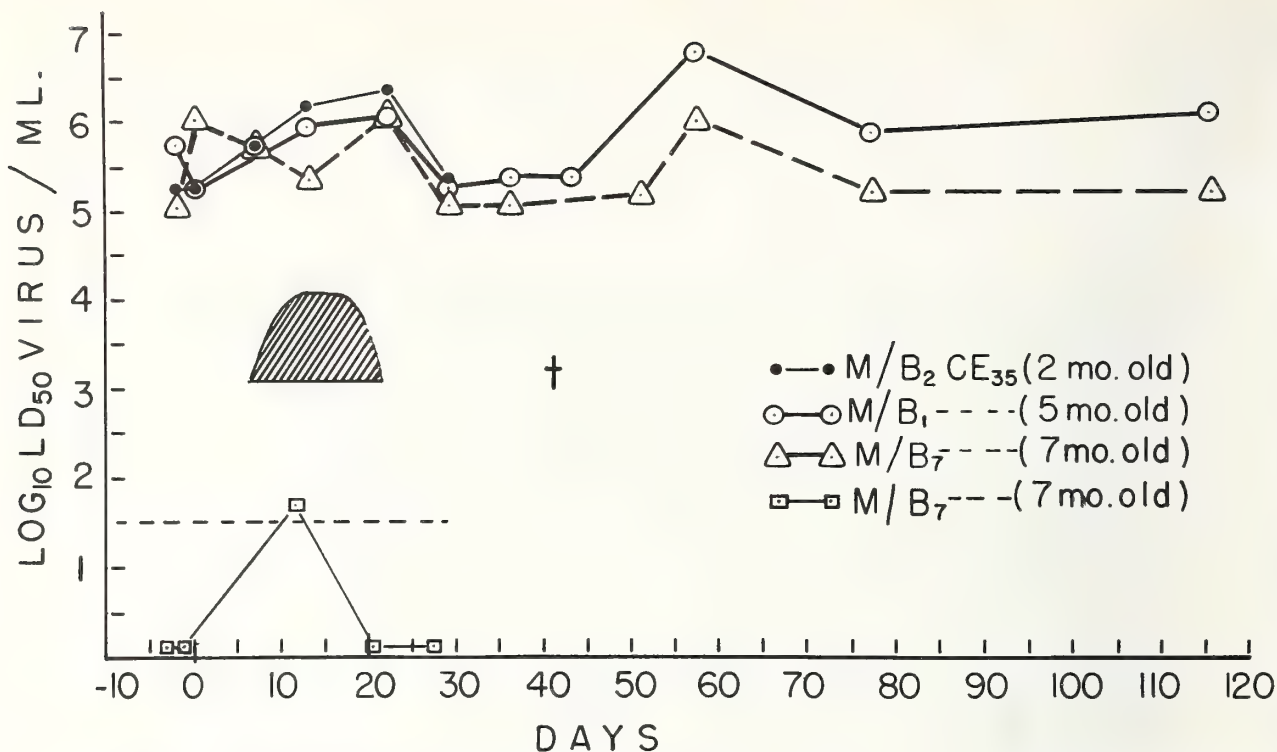


FIGURE 6.—Curves showing levels of virus in the blood and clinical disease in PTI mice before and after superinfection with *E. coccoides*. The shaded area represents the onset and duration of signs of disease, including ruffled fur, hunched posture, and blepharitis. Virus strains used and age at *E. coccoides* inoculation are shown for each mouse.

mice from each of three different pools, with the following histories: two had been made PTI at birth 2 months previously with a docile egg-passed LCM strain M/B₂CE₃₅, two made PTI 5 months previously with strain M/B₁ (curve C, fig. 4) and two made PTI 7 months previously with M/B₇ strain (curve B, fig. 4). All six mice were inoculated ip with *E. coccoides* after being bled on two occasions in order to determine their blood LCM virus titer; they were then checked daily and bled at frequent intervals to detect changes in virus titer of the blood. The results of this experiment are shown in figure 6. The main effect of the *E. coccoides* was to induce sickness in all the animals beginning on the fifth day and consisting of signs of acute LCM disease in adult mice—ruffled fur, hunched posture, and blepharitis—but not severe enough to cause convulsions. The titration results showed a rise in titer with a peak at day 21, and also a second rise at day 56. The first peak was different in the third mouse (M/B₇) and the variation in blood virus titer of this mouse before *E. coccoides* inoculation was as great as the magnitude of the rise in the other two. In view of this variation and the small number of animals used, little significance can be attached to the changes observed. Mouse No. 1 died as a result of an abscess

which developed at the bleeding site, and the other one from that pool died shortly after inoculation with *E. coccoides*; this was the youngest group (2 months) and it has been found in this laboratory that younger PTI mice are more susceptible to the effect of *E. coccoides* than older ones. The rise in titer of the fourth mouse (M/B₇) on day 12 is not so significant as it appears on the curve since the threshold of sensitivity of the titration procedure (indicated by a dotted horizontal line in fig. 6) is such that virus concentrations less than $10^{1.5}$ cannot be detected; thus, in this mouse there was insufficient virus present to cause more than a moderate probability of detecting virus at any time. However, the low level of viremia in this mouse should have made possible the detection of a significant rise if such had occurred; the absence of such a rise confirms the general conclusion that *E. coccoides* superinfection of PTI mice does not cause a significant increase in viremia, and that the temporary but fairly severe illness produced by it must be caused by some other mechanism than increased virus multiplication.

The Injection of Antiserum or Lymphocytes.—An experiment was made to see if the injection of hyperimmune anti-LCM serum or preparations of live immunologically active spleen cells would induce dis-

ease in PTI mice, or reduce the titer of the carrier virus. This experiment was based on evidence that LCM disease in adult mice is due to an immunological conflict in the infected animal, between its cellular immune response and the viral antigen; the role of humoral antibody seemed minimal since several workers (12, 13) have failed to find significant amounts of neutralizing antibody in the LCM immune mouse. It was found that ip injections of volumes up to 1 ml. of anti-LCM rabbit or monkey serum with high neutralizing titer, or serum from immune mice, had no visible effect on PTI mice. Several experiments were performed involving inoculations of immune cells into groups of 10 to 20 known PTI and normal mice using doses varying from 1×10^6 to 3×10^7 cells given iv, or 1×10^6 to 2×10^8 cells given ip to each mouse. At the time of these experiments only acute signs of LCM disease were sought and most of the experiments were terminated 3 weeks after the day of inoculation. No signs of disease were produced by this procedure, except in a few cases—those due to peritonitis or abscess formation consequent on failure of absorption of the larger cell inocula. In one experiment, PTI mice which had received 3×10^7 immune mixed lymphoid and thymus cells iv were retained for 6 weeks with repeated blood virus titrations every 4 to 5 days; these animals showed no signs of disease and their virus titer remained unchanged during the period.

At this time it was concluded that insufficient immune cells were available by injection to cause a clinically evident effect upon the PTI animal or to reduce detectably their virus titer; however, in the light of subsequent results by Volkert (14, 15) it seems likely that the experimental animals were observed for insufficient time after the immune cell injection.

The Effect of Parabiosis Between PTI and LCM Immune Mice.—In order to determine whether a larger quantity of immune cells would cause disease and suppress the persistent virus infection in PTI mice, experiments were performed in which parabiotic unions were made between PTI and LCM immune mice. The preliminary results of this study (8) indicated that late disease could readily be induced by appropriate parabiotic union. Initially parabiosis was attempted by cutaneous junction only, but this proved to be inadequate to maintain the union between vigorous animals, and the method was given up in favor of the much more satisfactory coelomic parabiosis which has the overriding advantages of strength and the formation of a common peritoneal cavity, which can effect extensive transfer of cells or antibodies. In the first experiment, which involved para-

biotic union between PTI and immune mice, the animals had been left over from other studies; subsequent experiment were made with fresh animals and attempts were made to eliminate any possibility of genetic differences in different litters causing heterogeneous disease by rendering the mice completely tolerant to each other by neonatal mutual cell inoculation as described under Materials and Methods. One third of the litters were then inoculated ic with 0.02 ml. of 10^{-2} strain M/B₆L₁₁ LCM virus to produce the PTI mice; the remainder were left until adult when half of them were immunized against LCM virus (as described under Materials and Methods); the other half were used as normal controls. At the time of the parabiosis operation, only mice of the same sex were joined and, apart from the first experiment, only females were used, to eliminate fighting between pairs. All PTI mice were tested for positive viremia before joining and were stained on the back with Bouin's solution to facilitate identification; in addition, the PTI animal was always made the left hand member of the pair. Only mice of comparable weight (within 1 gm.) were joined; usually animals weighed 16 to 22 gm at the time of operation. The results of all the parabiotic unions are summarized in table I. Union of pairs of normal control mice caused no signs of disease in any of the pairs (fig. 5 D and E) over a period of $3\frac{1}{2}$ months, indicating that the mice were fully tolerant to each other; three normal mice were joined to immune, the one operation survivor appearing quite normal $6\frac{1}{2}$ months afterwards (fig. 5F). The parabiotic PTI-immune mouse pairs showed six acute deaths in the first experiment occurring in the first 2 days after operation; apparently death occurred first in the PTI animal since several of the immune animals remained alive for several hours after the partner's death; the remaining pairs in this experiment either died from the anesthetic or had to be killed owing to failure of the operation wound to remain closed. The acute deaths were at first thought to be due to an anaphylactic reaction of the PTI mice involving the LCM virus antigen, and the antibody present in the immune partner; however, the effect was not evident in subsequent experiments involving PTI-immune unions, and therefore these deaths were ascribed to failures of the operative technic since they were the first series to be performed. Subsequent experiments showed a very low operative mortality (4/44) as experience was gained with the technic. Extremely interesting changes occurred in the subsequent parabiotic PTI mice joined to immune partners, occurring at different rates in different pairs, the earliest being

TABLE I.—Results of Parabiotic Union of Pairs of Mice of Differing Immune Status With Respect to LCM Virus

Type of mice in parabiotic pair	Number of pairs	Operation deaths	Immediate deaths ¹	Figure No.	Result
Normal/normal	11			5D 5E	All alive and perfectly healthy 120 days. ²
Normal/immune	3	1		5F	One died on day 10 of wound sepsis, one perfectly well photographed at day 195 (6½ months).
PTI/immune	25	9 ³	6	5G 5H 7A 7B 7C ⁴	Three PTI died after 27, 42, and 59 days. All showed ruffled fur, beginning day 4 to 8, continuing indefinitely. Seven remained alive but showed varying degrees of wasting, anemia, alopecia, hunched posture, ophthalmitis with onset between days 28 and 60.
PTI/normal	13	2	2 Normal ⁵		All pairs showed illness mainly in the normal mouse with onset on day 7 or 8. One PTI died day 7. Remainder: Normal mouse died days 8, 10, 16, 19, 29, 36. Only two pairs recovered; bleeding showed both to be PTI. These developed disease and died 4 months later.
PTI/PTI	6			7D	One pair died after bleeding. Four pairs appeared normal for 120 days; one pair showed wasting and anemia.

¹ Within the first 3 days.² Time intervals referred to in this column indicate days after parabiosis operation.³ Three died from the anesthetic; 4 were destroyed owing to

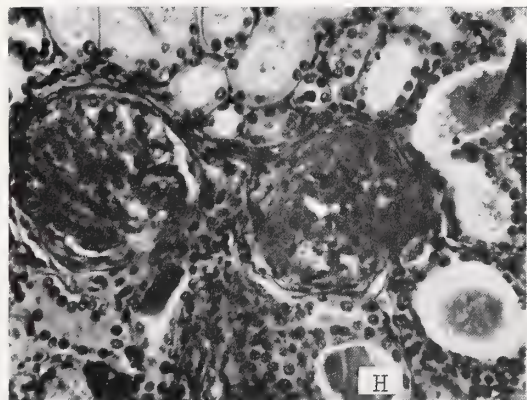
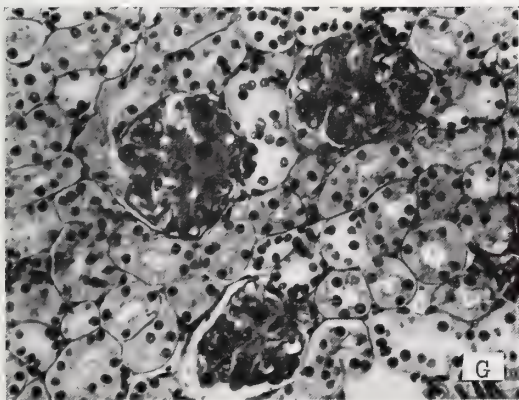
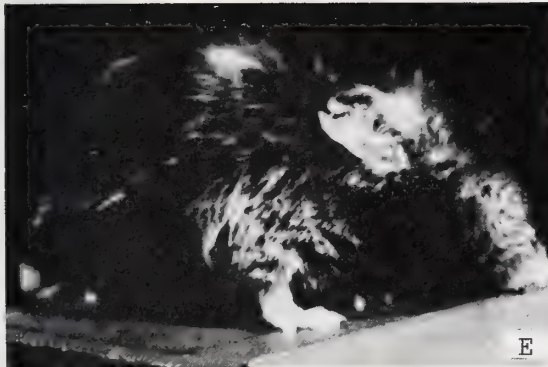
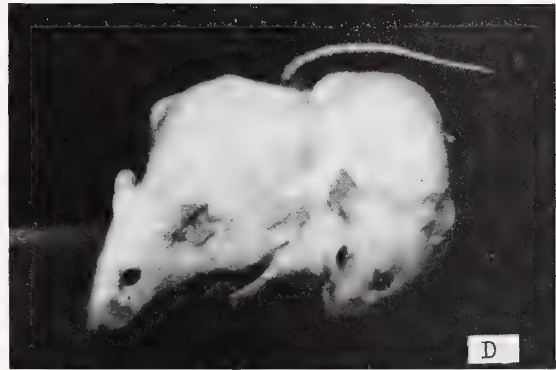
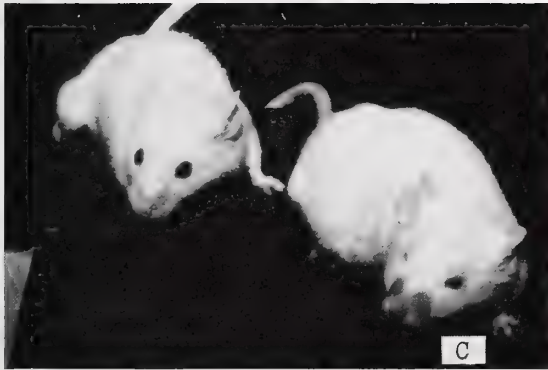
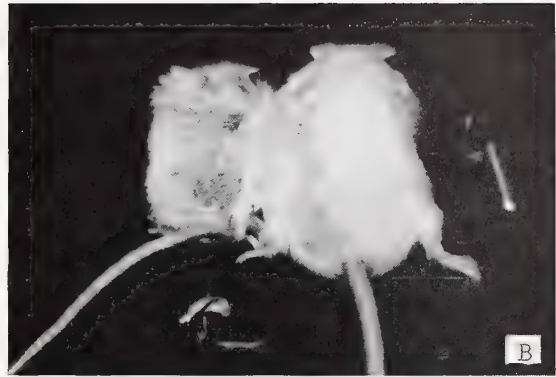
wound sepsis, 2 died as a result of orbital bleeding at 14 days.

⁴ Control pair, same history as others but not joined.⁵ Occurred on day 2, cause unknown.

noticed after 28 days. These changes were remarkably similar if not identical to those described earlier as the "late disease" of PTI mice; however, the onset in this case occurred as early as 4 to 8 weeks after parabiotic union which was usually performed when the mice were about 8 weeks old. Thus the onset of "late disease" in these mice occurred when they were only 3 to 4 months old compared to onsets after 7 to 18 months in nonparabiotic PTIs. PTI-immune parabiotic mice showing mild disease (ruffled fur and weight loss) are shown in figure 5G, and a pair in which the PTI mouse shows signs of severe disease or runting is shown in figures 5H and 7A; these changes, consisting of weight loss, hunched posture, ruffled fur, alopecia, extensive ophthalmitis, anemia (as judged by the color of the ears, skin, and tail vein), and a thin, hard, rigid condition of the tail (fig. 7B) all occurred in the PTI animals only, and in general the immune animals all appeared extremely well, apart from occasional slight ruffling of the fur. Comparable control PTI and immune mice with the same histories as the parabionts, but unjoined, are shown in figure 7C, at a time equivalent to 210 days after parabiosis.

PTI and normal mice were joined (13 pairs) and quickly showed a different picture, with the onset of typical acute LCM signs 7 or 8 days later; the major disease signs occurred in the normal mouse. All the normals except two died after 8 to 36 days, and blood virus titration of the survivors showed that both mem-

FIGURE 7.—A. PTI/immune parabiotic mice, same pair as in figure 5H, but photographed 20 days later (110 days after joining); the PTI mouse died the following day. Reproduced by permission from Cold Spring Harbor Symp., 27, 479, 1962 (8). B. Same as A showing wasting, hardness and rigidity of the tail of the PTI mouse. C. PTI and immune (control) mice with the same histories as those used for parabiosis, but not joined, showing normal appearance. Photographed 210 days (7 months) after the time when the parabionts were joined. The PTI mouse is on the left of the photograph. Reproduced by permission from Cold Spring Harbor Symp., 27, 479, 1962 (8). D. PTI/PTI parabiotic mice showing normal appearance 120 days (4 months) after joining. E. Alopecia in a runt mouse produced by transplantation of immunologically competent splenic cells into an immunologically neutral host. Reproduced by permission from Oliner, Schwartz, and Dameshek, Blood, 17, 20, 1961 (25), and Cold Spring Harbor Symp., 27, 479, 1962 (8). F. Parabiotic mice 45 days after operation. Animal in foreground is an F1 hybrid with characteristic wasting and skin lesions. Animal in background is a CBA mouse. Reproduced with permission from van Bekkum, Vos and Weyzen, J. Nat. Cancer Inst., 23, 75, 1959 (26). G. Section of kidney from a PTI mouse with well marked late disease showing diffuse hyaline thickening of basement membrane, accumulation of material on capillary basement membrane with partial to complete obliteration of lumen. Periodic acid-Schiff stain. Reproduced by permission from Nature, 203, 1357, 1964 (38). H. Same as G, showing hypercellular glomeruli, adhesions and epithelial crescent. The tubules are dilated and contain casts. Reproduced by permission from Nature, 203, 1357, 1964 (38).



bers of the pair were now PTI with blood virus titers of 10^4 LD₅₀/ml.; one of these pairs showed both mice to be small, ruffled, and anemic 4 months after joining; the other remaining pair was sacrificed.

Six pairs of PTI mice were joined of which one pair succumbed after bleeding and four pairs remained disease free for 120 days (and were then sacrificed) (fig. 7D); the final pair developed wasting and anemia after 63 days; the group was sacrificed at 120 days.

Weight Changes in PTI-Immune Parabiotic Mice.—Weight changes in LCM infected mice have been shown to bear a very close direct relationship to the severity of clinical disease (16). Two pairs of PTI-immune parabionts of which the PTI mice were very sick, were anesthetized, separated and their parabiotic tissue excised and the wound sewn up; this was somewhat difficult as intestines of both animals had

become intimately related with adhesions. The animals were then weighed and their weights followed for 90 days (3 months). These results and the weights of a third pair, which were weighed regularly but not separated, are shown in figure 8. After the parabiosis operation, at day 53 after birth and inoculation of the mice, the joined mice (19 gm. each) lost 3 to 5 gm. in weight which, after separation, was seen to be the result of slight gain in the immune, and marked loss in the PTI. After separation, one immune failed to recover from the operation but the other gained weight, rapidly approaching the control value for normal mice, whereas both separated PTI mice were only 18 gm. 95 days after separation, one mouse having slowly gained 4 gm. since separation, the weight of the other having remained constant; in both cases the PTI mice still weighed less than when they were

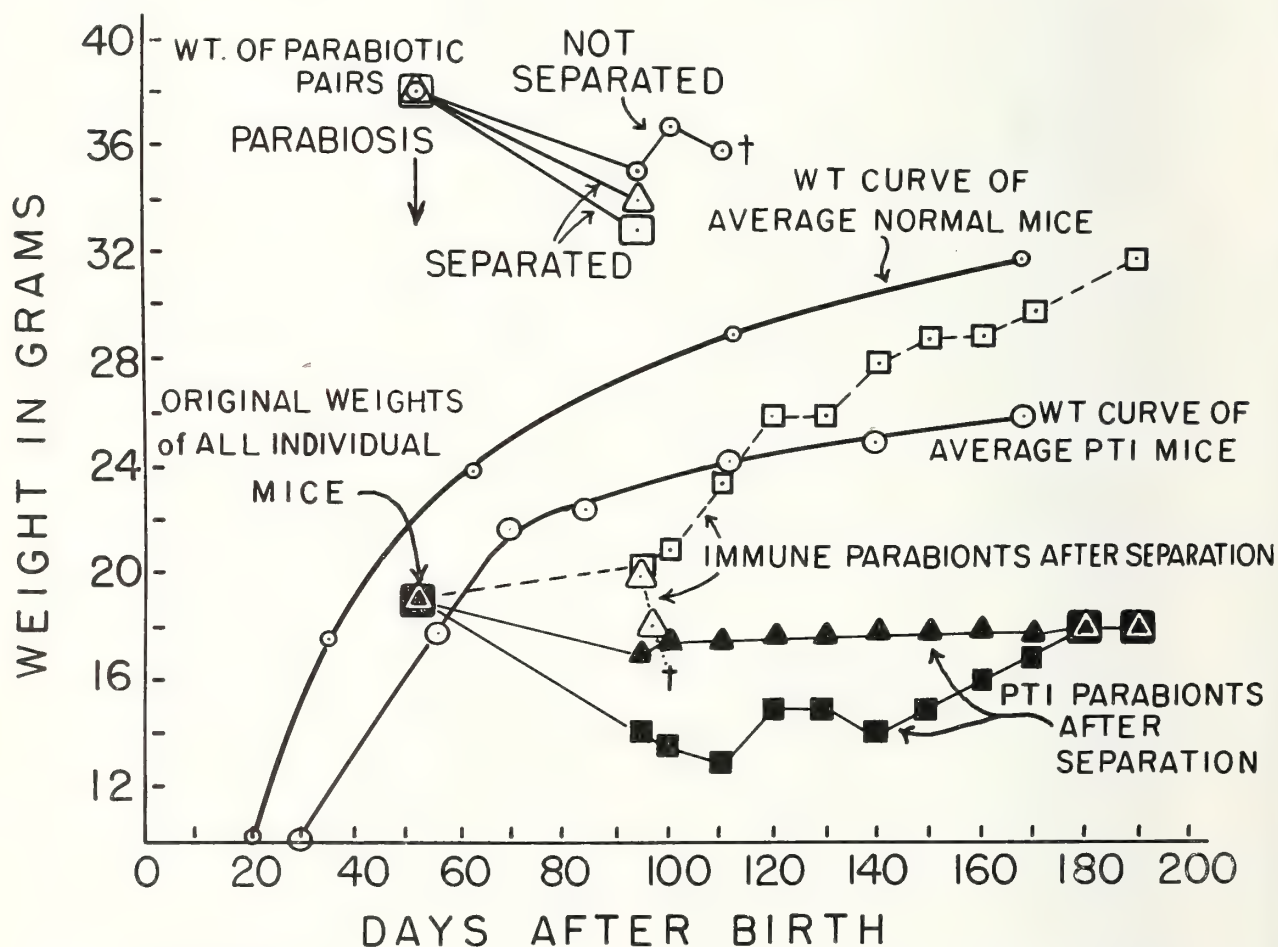


FIGURE 8.—Curves showing weight changes of 3 pairs of PTI-immune parabiotic mice, 2 of which were surgically separated 42 days after union. Both the individual mouse weights and the weights of the joined pairs are shown (weights after separation include the metal skin clips). Individual PTI mice after separation are shown with closed points and solid lines, and individual immune mice with open points and broken lines. For comparison, the average weight curves for PTI and normal mice are included (from fig. 3).

joined, and they continued to appear chronically sick without significant clinical improvement. This result indicated that the changes brought about by parabiotic union of PTI with immune mice were reversible by separation in the immune animal but not in the PTI animal.

Anemia and Virus Titer in PTI-Immune Parabiotic Mice.—To confirm objectively the clinical diagnosis of anemia in the PTI mice joined to immune partners, two pairs showing pale ears and tail in the PTI mouse were individually bled 23 days after being joined, and red cell counts were made on each sample. The results are shown in table II. In each case the

TABLE II.—Results of Red Blood Cell Counts on the Individual Mice in 2 PTI-Immune Parabiotic Pairs in Which the PTI Showed "Late Disease." One Pair was Then Sacrificed and Virus Titrations Made on the Liver and Spleen of Each Mouse. Both Pairs Were Joined on the 80th Day After Birth and Bled on the 103d Day, 23 Days After Parabiosis

Type of mouse bled	Red cell count ¹ millions/mm. ³	Liver titer LD ₅₀ /gm.	Spleen titer LD ₅₀ /gm.
PTI	4.0	10 ^{6.4}	10 ^{6.5}
Immune	11.6	10 ^{1.4}	10 ^{2.6}
PTI	2.7	NT	NT
Immune	8.9	NT	NT

¹ Normal range of rbc/cmm = $8-11 \times 10^6$, average 9.7×10^6 . NT = not tested.

rbc count was approximately 3-fold lower in the PTI animal than in the immune, which was close to the normal limits for mice, of $8-11 \times 10^6$ rbc/mm³ with an average of 9.7×10^6 rbc/mm³ (17). One of the pairs was sacrificed after bleeding and the liver and spleen of each mouse were removed and titrated for virus; the results are shown in table II. The PTI animal's organs contained more than 10⁶ LD₅₀/gm. whereas those of the immune mouse contained only about 10² LD₅₀/gm.; thus each PTI animal had 10,000 times the virus concentration of its immune partner. No virus could be detected by similar titrations of control immune mice. Portions of the spleens and livers were also sectioned and submitted for routine pathological study, but no significant findings were noted.

Blood counts were done on several symptom-free PTI and immune mice, which at first were thought to indicate very low counts. However, further study showed that the oxalated blood of these mice was clotting on contact with the Gower's solution used as di-

luting fluid, thereby preventing mixing of the cells. The factor responsible for this has not been determined, but the effect was not obtained when heparin was used as anticoagulant, and normal counts were obtained.

CONCLUSIONS AND DISCUSSION

The Relation Between Neonatal LCM Virus Infection and Autoimmune Disease in Mice.—The main feature which emerges from this work is the initiation of a syndrome of degenerative disease occurring late in life in mice which have been infected with LCM virus shortly after birth. At the present time none of the observed facts is at variance with the concept that this series of events is due to the early development of immunological tolerance to the viral antigen, followed by diminution of tolerance in later life, with the initiation of a gradual autoimmunological response resulting in autoimmune disease. This virus is known to be able to multiply in many different, if not all, tissues of the mouse as shown by these studies and previous work (12, 18, 19, 20) and consequently any immune process directed against it could be expected to be very widespread. LCM virus is not cytopathic in vitro to cells of several species including mouse embryo (4, 21, 22) so it would not be surprising if the virus were relatively harmless to tissue in vivo, as appears to be the case. On the other hand, this virus may well be able to cause an immunologically recognizable phenotypic mutation, or transformation of the cell membrane as is the case with influenza virus (23) and with polyoma virus (24). Under these conditions the immune response of the host against the virus would also be directed against the LCM infected cells.

The "late disease" of the PTI mice has clinical features in common with the LCM runs and with the disease induced in PTI mice by parabiosis with immune mice, these being mainly: weight loss, hunched posture, ruffled fur, blepharitis, and alopecia; in each case the disease is associated with an immunological conflict between host response and virus, in which the immune response is unable to quench entirely the virus activity. In the infant runs, the immune response fails or is repressed, producing tolerance, and in the older PTI mice the tolerance appears to be "wearing off" or possibly immunological "intolerance" increases with advancing age. It is evident that in the case of the PTI-immune parabiotic pairs the disease is produced in the PTI as a result of the close coelomic contact with an actively immune animal; such mice have suppressed LCM infection in themselves and under

the stimulus of transferred virus (which reached approximately 10^2 LD₅₀/gm. in the immune mouse tested compared with no detectable virus in control immune animals) would be expected to produce a strong immune response against the viral antigen of the partner to restore homeostasis. However, the failure to reproduce late disease or a similar runting condition in all of the PTI mice parabiotically joined to immune partners suggests that an additional factor may be involved. The most obvious candidate for this is minor immunological incompatibility between the pairs, a fairly likely event since the mice used were not a completely inbred strain. The experimental results of parabiosis between incompatible pairs has been well studied and is similar to the runting found in the parabiosis experiments described here. This condition has been described in detail by Oliner, Schwartz, and Dameshek (25) and a photograph of one of their mice showing severe runt disease is shown in figure 7E. The condition is essentially the same as "secondary disease" (26) or "homologous disease" (27) after protective foreign bone marrow injection of supraethally x-irradiated mice. The appearance of the lesions of the PTI mouse in a PTI-immune parabiotic pair is strikingly similar to those of the F1 mouse in an F1 parental hybrid parabiotic pair as described by van Bekkum et al. (26). A photograph of an example of the latter condition is shown in figure 7F by courtesy of these authors; the wasting

and skin lesions of the F1 mouse closely resemble those of the PTI mouse in figure 7 A and B. The fact that the runting in our experiments always occurred in the PTI animal indicates that either this animal was immunologically "LCM transformed" so as to be subject to rejection by the LCM-immune partner, or that its cellular regenerative capacity was impaired in such a way as to render it more susceptible to minor degrees of natural immunological incompatibility and parabiotic intoxication. The bulk of the parabiosis experiments indicated the latter conclusion and the occurrence of anemia in these animals but its absence from nonparabiotic PTI mice supports this view.

The status of the autoimmune and other possible mechanisms of LCM late disease will now be considered.

The Autoimmune Theory.—Differently produced types of murine immune disease are known to vary in respect to their symptomatology, and the main criteria of some of these are listed in table III with the comparable conditions described here induced by LCM virus. It is evident that a very close parallel exists between all these conditions; at the present time there is a strong circumstantial evidence that "late disease" is an autoimmune phenomenon resulting from the neonatal virus infection.

If "late disease" can be entirely attributed to virus induced autoimmune reactions of the host, LCM virus should probably be classed as an "autoallergenic" or

TABLE III.—A Comparison of the Clinical Signs of Immune Diseases in Mice

Criteria	LCM induced conditions:			Induced autoimmune diseases:		
	LCM-PTI runs	LCM-PTI late disease	PTI/immune parabionts	Autoimmune adult runs ¹	Secondary x-ray disease ²	Autoimmune infant runs ³
Mortality	0-100 varies with virus strain	100 per cent	Approximately 90 percent	Over 75 percent	Up to 100 percent	Varied + Mouse strains
Weight loss	+	+	+	+ Severe	+ Severe	+
Hunched posture	+	+	+	+		
Ruffled fur	+	+	+	+		+
Facial edema (blepharitis)	+	+	+	+		
Mincing gate	+	+	?	+		
Alopecia	+	+	+	+ In advanced stage	+	+
Anemia	NT	NT	+	+ Hemolytic		
Lymphoid hypoplasia	NT	+	NS			+ Extreme
Diarrhea	±				+	+
Lymphoid infiltration of kidney or liver		+	NS			+

+ = definitely present.

± = present in moderate degree.

NT = not tested.

NS = data not significant.

¹ Oliner et al. (25).

² van Bekkum et al. (26).

³ Billingham et al. (10).

"autoimmunogenic" virus. Burnet (28) has discussed the overall criteria of autoimmune disease using four autoimmune "markers" attributed to I. Mackay; these criteria include:

1. Is the serum γ globulin raised?
2. Are there significant numbers of lymphocytes and plasma cells in the disease process?
3. Are other clinically accepted autoimmune processes present?
4. Is there temporary or sustained benefit from anti-inflammatory corticosteroids?

If LCM disease, both acute and of late onset, is examined by these criteria, it is clear that in three out of four cases where the data are available the answer favors an autoimmune mechanism. Data are lacking on question 1 although preliminary results in this laboratory indicate that there is no significant change in the electrophoretic pattern of serum during PTI infection through the onset of late disease. To questions 2 and 3 the answer is unequivocally positive, and previous work (4) has shown the answer to question 4 to be positive. By current standards, therefore, the manifestations of LCM virus infection of mice prove to be acceptable but not proven as autoimmune disease. The causal role of the inoculum itself is inescapable since the disease did not occur in any normal control mice; the role of immunity in its causation is also likely from the ability of immune mice to induce a similar disease in PTI parabionts, apart from other evidence of the critical role of immune response to LCM virus (5, 8, 18) and the fact that agents such as x-rays overcome this and also exert a sparing effect on this virus infection (7). In the case of the parabiosis experiments, the possibility exists that heterologous disease might have arisen from genetic differences between the mice, or even as a result of the cellular injections designed to prevent this. Both these possibilities are minimized by the facts that the disease only occurred in the PTI animals and not in immunes, and not in any normal-normal pairs. However, at present the definite possibility remains that the PTI state may render the mice unusually susceptible to autoimmune processes which would otherwise remain subclinical.

In summary it may be said that while the autoimmune theory of LCM late disease remains a circumstantially attractive possibility, there is no clear cut evidence that this is in fact the mechanism. Other possibilities must therefore be considered; these include the following which will be considered in turn:

1. Chronic direct histiopathic effects of LCM virus;

2. Lesions due to a non-LCM viral agent contaminating the LCM stock and:

- a. Autoimmune mechanism;
- b. Histiopathic effect;

3. A nonviral agent contaminating the LCM stock and chronic infection leading to amyloid disease.

Chronic Direct Histiopathic Effects of LCM Virus.—This obvious though nebulous possibility demands a definite place in this list even though the virus appears to lack tissue destroying ability. The fact that LCM virus causes little evidence of *in vitro* CPE in many different cell types (4, 21, 29, 30) does not eliminate the likelihood of a direct destructive effect on tissue, particularly since a slow (c. 14 day) CPE does occur in chick embryo tissue (21) and a faster one in strain L mouse fibroblasts (31). The very long incubation period (7 to 12 months) involved in LCM late disease would appear not to depend on delay in the virus reaching the affected organ, e.g., kidney, which is known (32, 26) to occur early on in acute infection; it is more likely to be due to a long drawn out deleterious effect of continued infection upon the cell, plus the slow appearance of clinical signs resultant upon cumulative cell damage or loss to a point where physiological effectiveness is lost. It is well known that the physiological reserves of organs such as liver and kidney are very large, and it is likely that in the mouse no overt evidence of failure may exist until 90 percent or more of function is lost. Figure 9 shows a diagram of the relationship between the kinetics of growth of a hypothetical slow virus and the appearance of overt disease, where the signs depend upon a physiological function of which the organ concerned had a 75 to 90 percent reserve capacity. Here the percent involvement of parenchyma cells infected by the virus depends upon a complex interplay of many factors. Some of the more obvious of these will be briefly considered here; their interplay can be crudely represented mathematically in the following way:

$$P_t \propto \frac{t \cdot V_g}{K_I \cdot K_R \cdot [IF][AB]}$$

where

- P = Percent cells infected by time t
 V_g = Virus growth rate
 K_I = Virus inactivation constant
 K_R = Regenerative constant of cells
 $[IF]$ = Concentration of interferon
 $[AB]$ = Concentration of antibody to virus

If V_g is relatively slow, the rate of increase of P will be correspondingly slow, and P is clearly directly

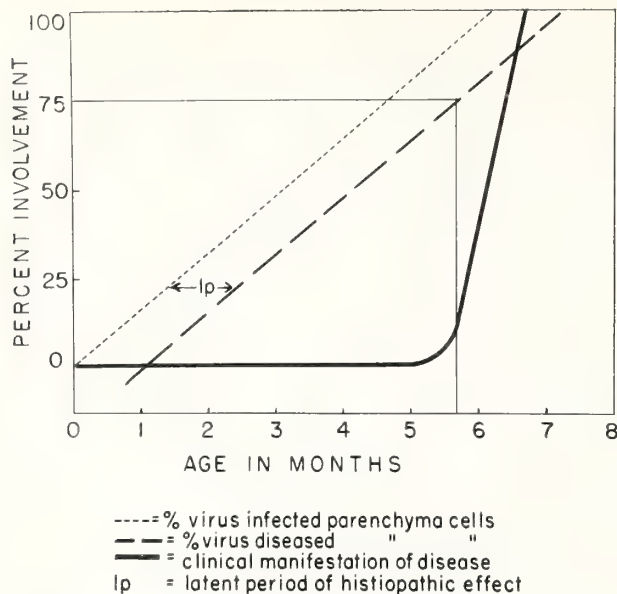


FIGURE 9.—Diagram relating percent cell infection, percent cell disease, and clinical manifestations of disease or death for a hypothetical slow virus involving a crucial physiological function with a 75-percent cellular reserve.

related to the stability of the virus and inversely to the degree to which the host cells can regenerate new uninfected progeny. If the system makes and is sensitive to interferon, the time to reach a high P value will be correspondingly prolonged; if a rising concentration of antiviral antibody occurs, the progress of a rising P value will be reversed, with ultimate eradication of the infection. Thus for a very gradual growth of slow virus over a period of weeks or months immunological tolerance or paralysis, or nonavailability of antibody, is a prerequisite; it seems possible that this may be the fundamental distinctive property of the slow virus group.

In figure 9 the percentage of diseased cells is shown displaced to the right from the infected cell curve by a distance *lp* representing the latent period of cellular pathology or the "histiopathic effect." This could well be several weeks in view of the observed 15 day LCM CPE in vitro, if it is assumed that in vivo conditions favor the prolongation of the normal structure of the cells. As the percentage of diseased cells rises to the critical value of 75 percent, sufficient diminution of function will occur for clinical signs of disease to appear; this is shown by the cutoff point indicated by perpendiculars in figure 9 and the sudden rise of the solid line representing clinical disease. The latter curve will reach 100 percent indicating severe illness or death during the relatively short time interval of

one month between 75 and 90 percent on the cell disease curve.

This type of mechanism can explain the long incubation period of late disease due to LCM in which progressive renal and probably other vital organ damage is involved, and can also be postulated to explain the sudden onset of disease due to scrapie and perhaps of such hypothetical virus diseases as kuru, multiple sclerosis, etc. The concept is also open to test by quantitative histopathological and other technics during the course of slow virus infections.

A Non-LCM Microbiological Agent Contaminating the LCM Stock Used.—The failure of superinfection of PTI mice with *E. coccoides* to precipitate clear cut acute or "late disease" indicates that the pathogenic mechanism of late disease differs from that of acute LCM, which has been shown (9) to become much more severe under these conditions. However, although *E. coccoides* is not a factor in the initiation of late disease it is possible that another murine parasite is involved in its pathogenesis. Other workers using long term LCM PTI mice have failed to observe the onset of any form of late disease (33, 34) even 18 months after infection. This suggests that either a contaminant or a special LCM variant is required to induce late disease. Evidence has been obtained in this laboratory that some inocula of UBC LCM virus stock have been contaminated with a PPLO which has been difficult to isolate and culture. Many of the mice inoculated at birth with such a stock LCM pool developed circling disease, a violent tendency to spin rapidly round and round. It appears that the PPLO which was isolated can reproduce this disease, but the evidence indicates that this disease is hard to induce except in mice which also carry LCM virus. It is at present too early to say if the PPLO is involved in the production of late disease; however this possibility is unlikely as only one of many LCM pools appears to be thus contaminated. It is noteworthy that Findlay (35) reported a similar form of synergism between LCM and a PPLO in causing spinning or circling disease of the middle ear in mice. Such double infection diseases are incompletely understood and need more investigation, particularly as concerns their human importance. Other microorganisms contaminating the virus stock might cause chronic infection leading to an amyloid type of degeneration which the renal lesions of late disease do resemble (36, 37, 38) as shown in figure 7 G and H. However, the practical possibility of a bacterial or other contaminant being present in the batches of LCM stocks used to produce late disease was checked by routine sterility tests. Since the

observation was made that liver tissue pools (but not brain) sometimes contained small numbers of a bacterial contaminant, all pools have been prepared in an antibiotic solution and prove to be sterile. The possibility still remains that the PTI state renders the mouse hypersusceptible not only to immune disease as evidenced by parabiotic experiments, but also to chronic degenerative effects due to its own bacteriological flora; however, even if this mechanism exists it is perfectly clear from the lack of late disease in mice inoculated at 4 weeks of age that the role of the congenital infection is paramount, and that furthermore a special strain of viral seed seems to be required.

However this disease is finally explained, the importance of this type of infectious entity and the group of slow viruses is firmly established. Very long incubation period diseases though mysterious and difficult to investigate may nevertheless operate by a straightforward and basically simple mechanism. It should be remembered that relatively long latent periods are not uncommon in the case of graft-induced immune disease; Oliner et al. (25) quote intervals of up to 100 days as latent periods in cell-induced runt disease in adult mice. All infectious disease can in a sense be looked upon as a titration between host defense and parasite virulence. In the slow viruses we are dealing with some gentle but deadly agents which cause very subtle destructive effects; in order to perceive these events more clearly, we must develop appropriately subtle indicators to detect and follow these new titrations.

SUMMARY

This paper describes experiments designed to explore the concept that the late onset disease occurring in mice approximately 12 months after neonatal inoculation with LCM virus may be due to an autoimmune mechanism. The long-term effects of persistent tolerant LCM infection on mortality rate and weight are described in detail.

Superinfection of LCM tolerant mice with *Eperythrozoon coccoides* caused a short period of acute sickness but did not appear to influence the course of the infection, in contrast to the marked effect of this agent on acute LCM infections.

Attempts to overcome tolerance of PTI mice with injections of antiserum or immune lymphocytes did not cause any rapid effects; long-term effects were not studied. Parabiotic union of tolerant and immune mice were studied fairly extensively. Some parabiotic tolerant animals showed an accelerated onset of a disease similar to "late disease" and also similar to other

forms of immune disease which are discussed. Union of tolerant mice with normal ones resulted in acute deaths of the latter. Union of tolerant to tolerant mice caused no effect, neither did union between normals or immune animals. Failure to obtain uniform disease in the tolerant mice parabiotically joined to immune animals seemed to indicate that the tolerant state rendered the animal more susceptible to minor immunological incompatibility between the two parabionts. Highly inbred mice were not used for this work.

Virological studies between PTI and immune parabionts indicated some depression of virus titer in the PTI and slight viremia in the immune which was 10^4 -fold lower than in the tolerant animal.

The sickness and wasting of PTI-immune parabionts was found to be reversible in the case of the immune but nonreversible in the case of the PTI. These results are discussed with respect to theories of autoimmune disease and possible mechanisms of pathogenesis of slow virus diseases.

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DISCUSSION

SEVER: The footpad reaction which you have described in mice with LCM is reminiscent of what we see in the footpad reaction in tuberculosis in mice. I wonder if you could describe the conditions under which you get this footpad reaction.

HOTCHIN: It is very simple. You inoculate the footpad with any desired dose of virus and after 7 or 8 days the footpad begins to swell up. The swelling lasts about 8 days with a peak after about 4 days. We find that in some mice, about 3 weeks after this, one cannot find the popliteal lymph node of that foot, whereas ordinarily one can easily find it; it seems to have disappeared.

KENYON: Do the mice with glomerular amyloidosis have normal electrophoretic patterns?

HOTCHIN: Yes, they have normal patterns, judged by a microtest. We have yet to do a more detailed study using larger volumes of serum.

JOHNSON: Can you get this positive footpad reaction with a killed virus, and are you carrying it out with the virus suspended in the same material as the animal was originally inoculated with? In other words, could it be a late hypersensitivity reaction rather than a direct infective action?

HOTCHIN: The answer to both questions is no. We cannot get a footpad response with dead virus and we have done all sorts of controls with normal brain tissue, putting it in the other foot, even using three

feet with different things in them. In fact, you can get a very sensitive neutralization test by mixing virus with antiserum, and we have used this method as a neutralization test. In general, we get much better neutralization if we do the test by peripheral inoculation rather than by brain inoculation. We have the strong feeling that brain is almost mysterious in being able to dissociate neutralized virus, and I know that other people have had a similar experience.

CASALS: This seems to be a very general rule with most neurotropic viruses and other animal viruses, that the peripheral neutralization test will allow detection of neutralizing antibody far beyond that which the intracerebral neutralization test will allow.

HOTCHIN: A further point about the footpad reaction is that if we use an anti-mouse-brain serum—virus mixture as a control for the neutralization test in the footpad, it exacerbates the reaction and we get a greater swelling than with virus alone, or with virus and normal rabbit serum, so that there are some odd things happening for which I do not have a ready explanation.

Rat Virus, an Agent with an Affinity for the Dividing Cell

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INTRODUCTION

The supplementation of the proceedings of a conference on slow, latent and temperate viruses by a discussion of rat virus (RV) infection may, upon first confrontation, seem out of place, particularly since this agent is best known for its rapid destructive action. On the basis of present knowledge dramatic late sequelae are considered to be due to irreparable tissue injury rather than to the persistence of the virus. This is widespread among both wild and laboratory rats (1), however, and may represent a potential source of error in animal virology studies. It is a recently discovered agent, and the spectrum of rat virus infection is not yet well known among laboratory workers. More importantly, a unique host-virus relationship is provided by the studies reported herein, offering a promising approach to the definition of the locus of action of a particular virus, using an *in vivo* system.

This study, including a review of the spectrum of rat virus disease, was presented a few days ago before the Association for Research in Nervous and Mental Diseases (2), hence will be presented here in an abbreviated manner and with a different emphasis. In adult animals rat virus is able to produce only latent infection, best recognized by the presence of neutralizing antibodies. Upon inoculation into neonates and sucklings severe and often fatal disease is induced. Sensitive and dependable morphologic criteria have been established for the recognition of this virus, both in tissue cultures, and in animal tissues. These include the appearance of intranuclear inclusion bodies, composed of virus particles, and subsequent irreversible cytopathic effects, eventuating in destruction of the involved tissues. Profound disturbances of growth and development are exhibited by animals which survive

inoculation made during the suckling stage. Among these are general indications of disturbed growth, such as runting and mongoloid state, interpreted as the result of widespread injury to immature tissues, with defective repair. More specific evidence of selective injury to germinal tissues is exemplified by the tooth dysplasia and the intractable cerebellar ataxia consequent to rat virus infection in the early postnatal period. The dental deformity is explicable on the basis of profound injury to odontoblastic tissue, which is capable of only abortive restorative activity (3, 4, 5). The cerebellar dysfunction results from the destructive action of the virus upon a germinal tissue which is totally incapable of regeneration. From these two latter manifestations of rat virus infection it is possible to postulate that the dividing cell is the prime target of rat virus. In the present report this thesis will be developed, using the attack of this agent upon the cerebellum to furnish a model of virus-cell interaction.

MATERIALS AND METHODS

The studies from which this report is derived were made primarily upon 188 intracerebrally inoculated neonatal and suckling hamsters. Concurrent studies were made upon 24 uninoculated control animals at parallel stages of growth and development. The agents used in these studies included RV strains 12, 13, 171, 312, and the LS strain. These inocula were derived from stock pools of RV, isolated in cultures of rat embryo tissues, cultured first in Eagles Basal Medium with 10 percent agamma calf serum, and maintained in a medium containing 2 percent agamma calf serum. It is stressed that this stock pool was in its original murine state and had not acquired increased virulence through previous animal passage. The source of the various strains of RV, and the technical

procedures for isolation, animal pathogenicity studies, serologic testing and titrations have been described in previous publications (1, 2, 6, 7, 8, 9).

RESULTS

Upon intracerebral inoculation of RV of suitable virulence into hamsters during the first 4 days of postnatal life severe and intractable cerebellar ataxia was induced in 119 of 129 animals, usually unattended by other manifestations of neurologic deficit or disturbed growth. Delay of inoculation until 5 to 7 days after birth resulted in cerebellar disease of lesser severity, affecting 10 of 20 animals. Inoculation at 8 to 11 days post nately produced no visible evidence of cerebellar disease in 19 of 20 animals; a few viral inclusion bodies were seen in the single exceptional sample. The cerebellar dysfunction resulted from a remarkably selective destructive action of RV upon the external germinal layer of that structure. The attack of RV proceeded as a single fulminant wave of cell destruction, manifested by the appearance of intranuclear inclusion bodies towards the end of the first week following inoculation, and succeeded within 2 to 3 days by irreversible cytopathic effects. As a result of this viral

action the tissue destined to form the definitive granular layer of the cerebellum was eliminated, depriving this organ of its major input station, and leaving it incurably crippled. The accompanying illustrations present key features of this viral action (figs. 1 to 6). Contrasting strikingly with the severe involvement of the external germinal layer evidence of direct viral action upon the Purkinje cell layer was essentially absent. The resultant picture was that of a postnatally induced profound hypoplastic state—a granuloпрival hypoplasia of the cerebellum in which the Purkinje cell remained as the dominant cortical neuronal element.

Although the viral attack upon the cerebellum constituted the salient feature in these studies, other sites in the central nervous system and in the somatic organs were also involved, but to a far lesser degree, however. A variable and sometimes heavy involvement of the subependymal cell plate, and of neuroglial cells migrating out from this tissue occurred. A few viral inclusions were observed in the choroid plexus, ependyma, meningocytes, meningeal and parenchymal vascular endothelium, and pineal gland. In somatic organs in-

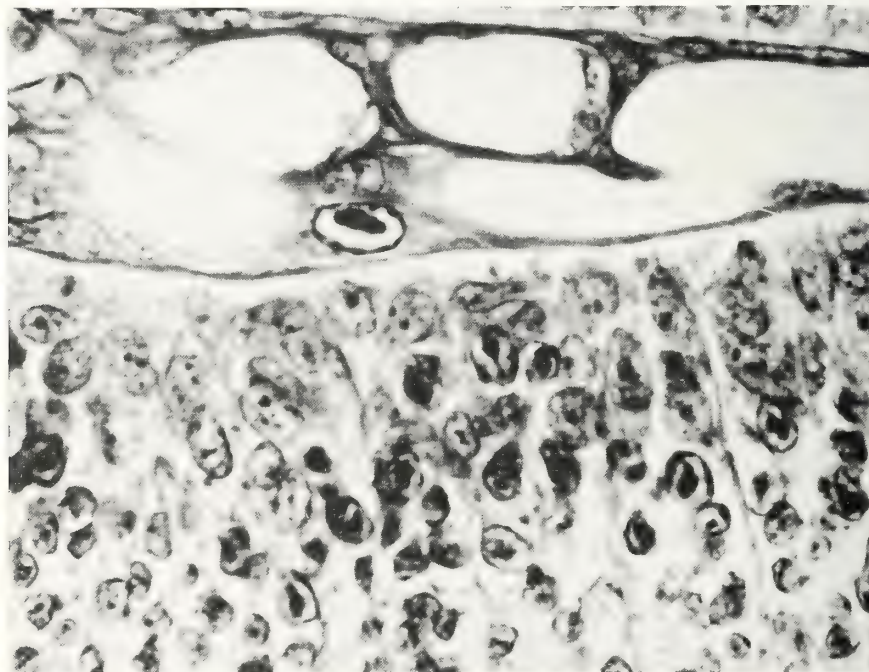


FIGURE 1.—Rat virus infection of cerebellum, inclusion body phase. A section of the external germinal layer showing partial involvement has been selected, so that inclusion bearing nuclei may be contrasted with normal nuclei. An involved meningocyte demonstrates the inclusion body phase particularly well. The characteristic dense central body, the surrounding halo, and the condensation of the nuclear membrane are illustrated. H. & E. $\times 800$

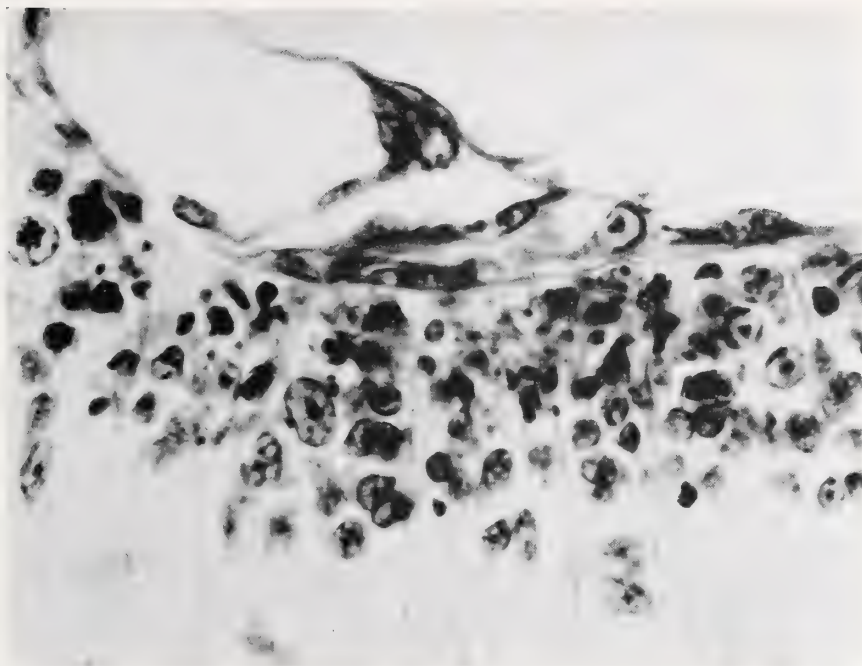


FIGURE 2.—Rat virus infection of cerebellum, cytolitic phase. External germinal layer showing severe cytopathogenic effects, involving most of the pictured cells. A few inclusion bearing neuroblasts and an involved meningocyte still show intact nuclei. H. & E. $\times 800$

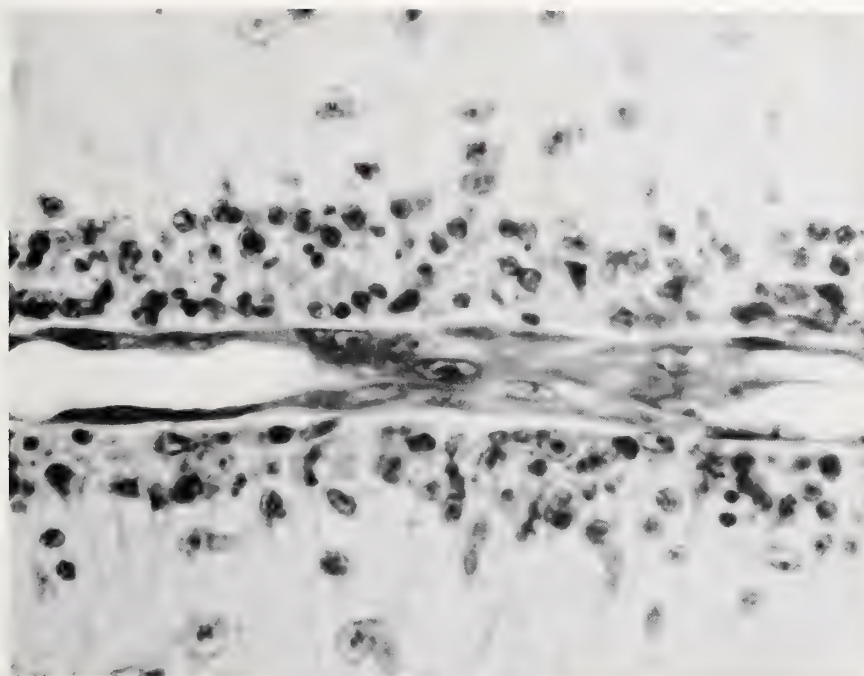


FIGURE 3.—Rat virus infection of cerebellum, cytolitic phase, late. External germinal layers, bordering a sulcus, showing virtually complete destruction of neuroblasts, with resultant severe depletion of this cell population. The few remaining viable cells in this layer bear inclusions. H. & E. $\times 625$

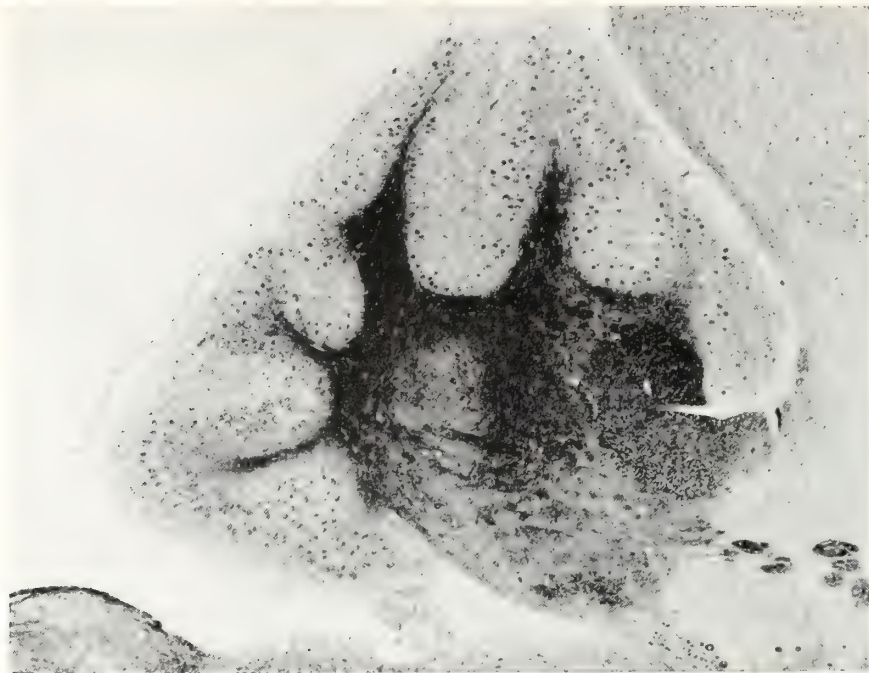


FIGURE 4.—Rat virus infection of cerebellum, hypoplastic sequel. Sagittal section demonstrating essentially total deprivation of the definitive granular layer as a result of destructive action of rat virus upon the external germinal layer. Normal white matter and deep nuclear structures are illustrated. Klüver-Barrera stain. $\times 40$

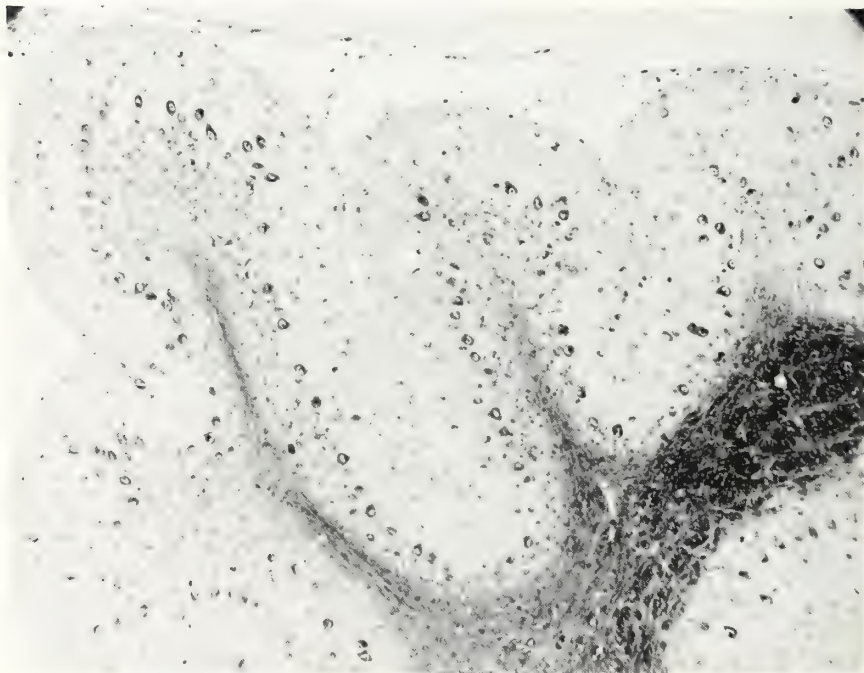


FIGURE 5.—Rat virus infection of cerebellum, hypoplastic sequel. Detail of cerebellar cortex demonstrating granuloprival state. Klüver-Barrera stain. $\times 120$

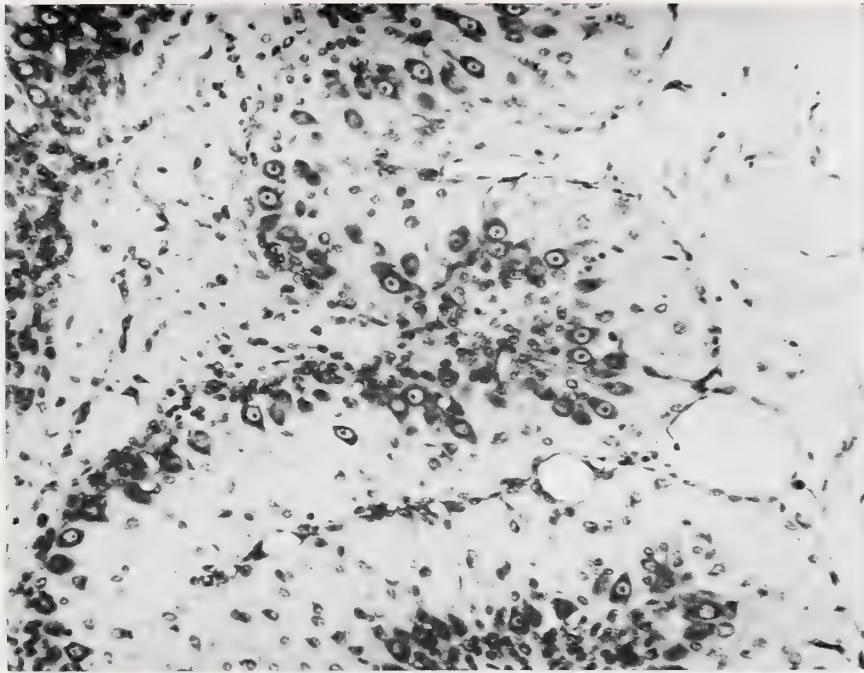


FIGURE 6.—Rat virus infection of cerebellum, hypoplastic sequel. Detail of cortex exemplifying granulooprival state and prominence and irregular disposition of Purkinje cell layer. Cresyl Fast Violet. $\times 250$

clusion bodies were found in the tooth germ, in myocardial cells, developing bone and cartilage, in pulmonary, hepatic, adrenal, and renal epithelium, and in endothelium, macrophages and fibroblasts of the liver, kidney, and spleen, in Schwann cells of peripheral nerves and in vascular smooth muscle. Squamous, but not glandular mucosal involvement and heavy involvement of smooth muscle of the gastrointestinal tract were observed in the inclusion body stage. Irreversible cytopathic changes observed at these somatic sites were not conspicuous, except for the tooth germ and skeleton.

DISCUSSION

The single feature common to all sites of viral involvement in these studies is the presence of mitotic activity. Cell proliferation is readily observable during the suckling stage in the tissues of control animals wherever intranuclear inclusions have been encountered in inoculated animals. In organs where division of relatively mature cells gives rise to similar daughter cells, i.e., the liver and heart, this relationship is suggestive of a relationship between viral susceptibility and proliferative activity. A firmer correlation is possible at sites of differentiating tissue such as the neonatal zone of the kidney, and the tooth germ furnishes

incontrovertible evidence of the affinity of RV for the dividing cell. The neonatal cerebellum, which presents a cell population high in mitotic activity in apposition to a post mitotic cell population offers perhaps the finest example of the propensity of RV to attack the mitotic cell. The major components of the adult cerebellar cortex are derived from two anlagen, each with distinctive and separately phased growth periods (10, 11). The Purkinje cells and deep cerebellar nuclei migrate radially from the primitive ependymal zone to their ultimate position, and undergo their final cell divisions prenatally. The external germinal layer migrates tangentially over the surface to occupy its superficial neonatal position. Late in gestation it initiates a phase of remarkable proliferative activity extending through a postnatal period of two or three weeks in various small laboratory animals. The replicative activity of this anlage is associated with an active inward migration, to form the definitive granular layer of the cerebellum as the external zone becomes depleted (11).

The severe attack of RV upon the cerebellum, the selective destructive action upon the external germinal layer, and the consequent blighting of the granular layer provide a ready means of following the active

phases of the infection. From observations of the early stages of this reaction a progressive sequence of events is postulated. Once the cerebrospinal fluid pathways have been cleared and the initial viremia has ended, the small anlage of the cerebellum, the external germinal layer, has been invaded. Exposure to the intracerebrally inoculated virus has been heavy, possibly involving this entire cell population, just at the time of initiation of its rapid phase of postnatal growth. The virus remains intracellular, replicating in parallel with the invaded cells, passing directly into daughter cells during mitosis and, until cell lysis occurs, spreading locally only by means of cell migration. Proliferation and migration of the virus-bearing cells proceeds normally despite the metabolic load created by the parasitism. With the development of the inclusion body phase of the infection virtually every cell of the now heavily populated external germinal layer may be affected. In this instance RV, in its inclusion body phase, may be looked upon as a marker of mitotic activity which by its replication has prevented dilution of the label, a disadvantage suffered by nonreproducing markers such as tritiated thymidine in the tracing of distant progeny of a cell population. The destruction of the external germinal zone by viral action, coupled with the inability of surviving cells to undergo reparative hyperplasia results in a hypoplasia of the cerebellar granular layer and enables one to reconstruct, even at periods remote from the active viral attack, details of the initial infection, including age of onset and severity of involvement.

While the above interpretation of virus-cell interaction appears valid it is manifest that further studies are required to support it. That this work is at an early phase is attested by the fact that the authors initiated their joint study only a year ago, a period considerably shorter than the incubation period of the class of viruses which forms the theme of this conference. To explore further this agent-host interrelationship the study is being broadened to include a more intensive survey of sites of tissue proliferation in the neonatally infected animal, and the use of fluorescent antibody and electron microscopy studies. In view of the lack of involvement of actively proliferating structures such as the glandular mucosa of the gastrointestinal tract and of hematopoietic tissues the role of differential tissue susceptibility to RV must be explained, if the postulated virus-cell interaction is valid. Additionally, critical studies exploring the relationship between cytodifferentiation and viral susceptibility, and investigating more precisely the association of viral replication and

DNA synthesis are required. Finally, the provocative and significant problems of delayed action of RV, of latency and reactivation of this agent, and the potential oncogenic and oncolytic effects of this virus, whose target appears to be the mitotic apparatus, remain to be investigated.

SUMMARY

Rat virus is presented as an agent having a selective affinity for the cell in mitosis. This virus-cell interaction is illustrated by the preferential attack of RV upon the external germinal layer of the neonatal cerebellum. The need for studies to confirm this postulated virus-cell interaction is stressed.

ACKNOWLEDGMENTS

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DISCUSSION

Q. Has this agent been put into rat tumors and adult animals to see whether or not it has an effect on other dividing cells?

MARGOLIS: We wondered about this and are doing something like this in two ways. We are trying it with polyoma, for example, and so far we have not had anything encouraging. Secondly, we are cutting out three-quarters of the liver of adult animals and inoculating the virus intravenously and watching whether it will appear in the regenerating liver.

SEVER: In view of the small defective skulls and the mongoloid appearance of the affected new-born animals, I wondered if the size of the cranium, *per se*, is likely to affect the pathological findings?

MARGOLIS: I do not think that the skull defect restricts the growth of the brain. For example, we find plenty of cerebrospinal fluid space, sometimes even what appears to be an external hydrocephalus, associated with malformed or hypoplastic brains.

SEVER: Since there have been reports of isolating this from human material, we were interested in the possibility that it might be involved in defects in humans. In a series of several hundred pregnant women, whose serum we had taken during pregnancy, we only found two who had antibody to rat virus and this by HI; and of these one had a mon-

goloid child, which was most interesting because of the association of the skull defects and also the chromosome defect in this disease. The other child is now 2 years old and appears perfectly normal at this point.

BANG: Have you found evidence of the virus in the intestinal epithelium, which is considered to be one of the fastest growing tissues in the body?

MARGOLIS: I have not found it in the glandular epithelium of the intestine yet. We were puzzled about this and wondered whether the turnover is so fast that one would not see the inclusion bodies. It may take 4 to 7 days for the inclusion body phase to appear and the cytopathic effect then occurs within 48 hours. I do not know whether this speculation is right or not, but the viral inclusion bodies can be seen in widespread sites.

KIBRICK: Is fecal material infective? If this agent was growing in the intestinal epithelium, then you might expect the feces to be infective.

MARGOLIS: I cannot answer that absolutely. We know that feces and urine together are infective, which makes the feces difficult to assess.

HABEL: Have you ever done any antiviral fluorescent antibody studies on infected cells that might be undergoing mitosis, to see if there is any association with the mitotic apparatus?

MARGOLIS: This is one thing we hope to do.

Latency of Infections with Simian Virus 40 and Adenovirus, Type 12

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The known animal tumor viruses often differ widely from each other. They may differ in size, morphology, stability, growth characteristics, type of nucleic acid, host range, and kinds of tumors induced. All the animal tumor viruses have two properties in common: (1) they induce tumors in susceptible animals, and (2) they are all capable of remaining in their hosts for long periods of time during which no signs or symptoms of disease are evident. The latter property is also common to some of the nononcogenic viruses and some pathogenic bacteria, and it is this property which is of concern at present.

Both simian virus 40 (SV-40) and adenovirus type 12 are examples of DNA viruses which are capable of inducing tumors at the site of inoculation when sufficient virus is injected into newborn hamsters. Apparently, a portion of either virus is capable of becoming assimilated into the host cells and of being replicated when the cell divides. These so-called "transformed" cells on transplantation into normal hamsters multiply and cause tumors which eventually kill the animals. If, however, the recipient hamsters have been immunized prior to transplantation with the virus which transformed the cells, then tumors do not develop or they develop more slowly than in the non-immunized hamsters (1, 2, 3).

The age of the hamster is important when tumors are induced by virus. Newborn hamsters are most susceptible. Susceptibility to SV-40 decreases with age up to 22 days (4). Susceptibility to adenovirus type 12 also decreases with age, and the oldest hamster reported to develop a tumor was infected when 14 days of age (5). Age is of little importance in transplantation experiments (6). Tumors develop in either old or young animals and the time required appears to depend on the number of tumor cells transplanted.

Large numbers of tumor cells cause tumor development in a short time, often within a few days, while the injection of small numbers prolong the period of tumor production (4).

Virus can usually be recovered from subcutaneous SV-40 virus-induced tumors but rarely from transplanted tumors. The amount of recoverable virus is usually small in virus-induced tumors, and cell-free extracts from such tumors contain insufficient virus to induce tumors when given to newborn animals (4).

To date, my coworkers and I have been unable to recover adenovirus type 12 from a single adenovirus type 12-induced hamster tumor.

Either virus will grow in different cell cultures with varying results. SV-40, for example, grows in cercopithecus monkey kidney cell cultures and produces characteristic cytopathic changes and finally complete destruction of all the cells. In rhesus monkey kidney cell cultures, virus is released into the nutrient fluid but none or only a few cells show vacuolization and ultimate cell destruction unless kept for approximately 30 to 70 days (7). In primary human kidney cells and other human tissue cells the infectious virus tends to disappear and rapidly growing, piled-up, bizarre "transformed cells" replace the normal cell sheet, as described by Schein and Enders (8) and by Koprowski and his group (9). On the other hand, two continuous lines of human tumor cells, HEp 2 and HeLa, behaved quite differently, as did primary chick embryo cells (10), either infected with leucosis virus (11) or free of it. All of these cultures remained viable yet released virus into the nutrient fluid.

HEp 2 cells in 2-ounce prescription bottles were infected with varying tenfold dilutions of SV-40, as shown in table I. The cultures appeared normal and the nutrient fluids were replaced at weekly intervals.

TABLE I.—Release of SV-40 From HEP-2 Cells Infected With Different Dilutions of SV-40 and Kept for 484 Days With Changes of Nutrient Fluid at Weekly Intervals

Incubation time of HEP-2 cultures <i>Days</i>	Dilutions of virus used as inoculum									
	Undiluted	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹ None
15	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	0	0	0	0
57	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	0	0	0
178							² 10 ^{6.0}			
332							10 ^{6.5}			
484										

¹ Only undiluted fluid tested.

² Assayed by production of CPE in cercopithecus monkey kidney cells using 2 tubes per dilution. Results are expressed as virus concentration per 0.2 ml. of fluid.

After 15 days, transfers of fluid were made from each bottle to cercopithecus monkey kidney cell cultures. The cultures inoculated from the HEp 2 cell cultures which had been infected with undiluted SV-40 and with SV-40 diluted 10^{-1} through 10^{-5} all produced characteristic vacuolization, and finally destruction of the cell sheet. After 57 days incubation, fluids from the culture were again tested for the presence of virus. Virus was present in fluids from all the cultures which had previously been shown to be producing virus and in addition, fluid from the culture infected with virus diluted 10^{-6} contained virus. The fluid from this latter culture was titrated in cercopithecus monkey kidney cell cultures on days 178 and 332, and titers of 10^6 and $10^{6.5}$ per 0.2 ml. were obtained. Even higher titers were obtained in fluids from the culture infected with undiluted SV-40 178 and 332 days earlier, $>10^{8.5}$ and $10^{7.5}$ per 0.2 ml. The HEp 2 cells did not flourish as well as the HeLa cells with a change of nutrient fluid only at weekly intervals and the cultures were finally lost.

The first HeLa cells were infected with undiluted SV-40 and with SV-40 diluted 10^{-1} through 10^{-5} on November 1961, over 3 years ago. The infected cells appeared to be normal although after a week or two, small spaces appeared between the cells which had been infected with undiluted SV-40 or with SV-40 diluted 10^{-1} . Soon the spaces were filled in with cells and one by one the cells in the bottles infected with the succeeding dilutions of virus also showed spaces between the cells which were soon filled in to form a solid sheet. The new cell sheets appeared to be uniform and heavier than the control noninfected HeLa

cells. The control cultures exhibited bizarre, piled-up cells typical of transformed cells. From time to time some of the cultures were lost due to bacterial or fungal contamination. For approximately 2 years, the nutrient fluids were replaced in the remaining cultures only once a week. By this time, the cell sheet appeared ragged and many cells were coming off the glass so nutrient fluid changes were made twice a week. A few weeks ago, the remaining cultures became contaminated and it is questionable whether they can be kept much longer. Mycostatin¹ appears to hold the contaminant in check, but cell multiplication proceeds slowly when it is present. The virus concentration in fluids from some of these cultures tested over a long period of time is shown in table II.

This was not an isolated experiment. A second lot of HeLa cells were infected with SV-40 in January 1962, and essentially the same results were obtained. The HeLa cells infected with undiluted SV-40 or with low dilutions of SV-40 first appeared as normal cells with small spaces between them. Next, cells grew into the spaces to form a solid uniform sheet of cells. The control cultures or the cultures infected with a low multiplicity of SV-40 grew as patches of bizarre transformed cells. The concentration of virus in the nutrient fluids of some of the cultures is shown in table III.

Since the concentration of SV-40 in some of the HeLa cell cultures was higher than in cercopithecus monkey kidney cell cultures, pools of four different strains of SV-40 were started in these cells, using a 10^{-3} dilution of virus for their inoculation in order to

¹ Made by E. R. Squibb & Sons, New York.

TABLE II.—Release of SV-40 From HeLa Cells Infected With Different Dilutions of SV-40 and Kept for 1,100 Days With Changes of Nutrient Fluids at Weekly Intervals for the First 2 Years and at Twice Weekly Intervals Thereafter

Incubation time of HeLa cultures Days	Dilutions of virus used as inoculum						
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	None
15	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	0
36	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	
77						² $10^{6.5}$	
120						$10^{9.0}$	
241						$10^{8.5}$	
395						$>10^{10.5}$	
547						$>10^{10.5}$	
704			$10^{8.0}$				
1,100			$10^{7.5}$		$10^{5.5}$		

¹ Only undiluted fluid tested.

² Assayed by production of CPE in cercopithecus monkey kidney cells using 2 tubes per dilution. Results are expressed as virus concentration per 0.2 ml. of fluid.

TABLE III.—Release of SV-40 From HeLa Cells Infected with Different Dilutions of SV-40 and Kept for 1037 Days With Changes of Nutrient Fluids at Weekly Intervals for the First 2 Years and at Twice Weekly Intervals Thereafter

Incubation time of HeLa cultures <i>Days</i>	Dilutions of virus used as inoculum										
	Undiluted	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	None
15	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	0	0	0	0
57	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	Undiluted ¹	0	0	0
178	2 10 ^{8.5}						10 ^{9.5}	10 ^{9.2}			
332	10 ^{8.0}							10 ^{8.5}			
484							10 ^{10.0}				
681	10 ^{8.5}										
1,037	>10 ^{9.5}										

¹ Only undiluted fluid tested.

² Assayed by production of CPE in cercopithecus monkey kidney cells using 2 tubes per dilution. Results are expressed as virus concentration per 0.2 ml. of fluid.

obtain a large amount of virus for other studies. The nutrient fluids were replaced at weekly intervals as before and the virus in the fluids was determined at 7- or 8-day intervals. The results are shown in table IV. Again, high concentrations of SV-40 were obtained.

TABLE IV.—SV-40 in Fluids From HeLa Cell Cultures Infected With Different Strains of SV-40 and Kept up to 103 Days With Changes in Nutrient Fluids at Weekly Intervals

Incubation time of cultures Days	Strains of SV-40			
	R426-6	A1685-5	A1385-5	H1385-5
14	¹ 10 ^{6.0}	10 ^{5.5}	10 ^{7.0}	10 ^{4.5}
21	10 ^{7.0}	10 ^{7.0}	10 ^{4.5}	10 ^{4.5}
28	10 ^{6.5}	10 ^{6.5}	10 ^{5.5}	10 ^{5.5}
35	10 ^{8.0}	10 ^{7.5}	10 ^{6.5}	10 ^{6.5}
42	10 ^{8.5}	10 ^{9.0}	>10 ^{9.2}	10 ^{6.5}
49	>10 ^{9.5}	10 ^{9.0}	10 ^{7.5}	10 ^{8.0}
56	10 ^{9.0}	10 ^{8.5}	10 ^{7.5}	>10 ^{9.5}
63	>10 ^{9.5}	10 ^{9.0}	10 ^{8.0}	10 ^{8.0}
70	10 ^{8.5}	10 ^{8.5}	10 ^{7.5}	10 ^{8.0}
78	>10 ^{10.5}	10 ^{8.5}	10 ^{9.0}	10 ^{9.5}
85	10 ^{8.0}	10 ^{8.5}	10 ^{9.0}	10 ^{8.0}
103 ²	Fluid	>10 ^{9.5}	>10 ^{9.5}	>10 ^{9.5}
	Cells	>10 ^{11.5}	10 ^{11.0}	10 ^{11.0}

¹ Assayed by determining the highest dilution of fluid which caused CPE in cercopithecus monkey kidney cell cultures using 2 tubes per dilution. Results expressed as amount of virus per 0.2 ml. of fluid.

² Fluid and cells were tested separately. Cells were removed from glass with versene and concentrated 40 to 50 times by centrifugation.

No effort was made to eliminate virus from these cultures or to do other studies which seemed indicated. Other HeLa cultures have been infected with SV-40 so that further work can be done.

In primary chick embryo cells the virus titer remained at a low level, <10¹, and the infected cells could not be distinguished from noninfected cells. The virus continued to be released from the cells for 178 days, the time of the last test.

Adenovirus type 12 also behaves differently in different cell cultures. In HeLa cell or monkey kidney cell cultures the virus titers, as determined by cytopathogenicity, are low and variable, often only 10^{2.0} to 10^{2.5} per 0.2 ml., but virus propagated in these cultures may have titers of 10^{5.5} to 10^{6.5} or even 10^{7.0} when tested in human embryonic kidney.

There is no explanation at present as to why some infected in vitro cell cultures yield infectious virus while others become transformed and do not yield

infectious virus. Certain factors which determine whether the virus will induce tumors in hamsters are known; age is one. It has been mentioned that young hamsters are more susceptible than old hamsters. Hamsters >3 to 22 days of age when infected with SV-40 develop tumors, but the numbers are reduced and the incubation time for tumor development is longer as age increases. Many older hamsters were infected with SV-40 in my laboratory but no tumors developed. Adenovirus type 12 injected into pregnant hamsters soon after mating also failed to induce tumors and tumors did not develop in the offspring with the exception of one animal.

The concentration of virus used to infect the newborn hamsters is a second determinant of tumor induction. SV-40 having a titer of 10^{7.5} per 0.2 ml. was diluted in 100-fold steps and used to infect newborn hamsters. The greatest dilution of virus which induced tumors in all, or almost all, of the hamsters injected was only 10⁻², and they developed tumors somewhat more slowly and lived longer than hamsters which had received undiluted virus. Virus diluted 10⁻⁴ induced tumors in only a few hamsters, and these appeared after a prolonged incubation time. Hamsters infected with SV-40 sometimes develop tumors near the end of their life span.

Although the concentration of virus used to infect the newborn hamster does influence tumor induction, the average incubation time appears to be a property of the virus. Thus hamsters infected with SV-40 when newborn usually begin to develop tumors 100 days later although occasionally a few tumors have been seen as early as 70 days after infection. Adenovirus type 12-induced tumors usually begin to appear about 33 days after infection, occasionally as early as 26 days or at late as 153 days.

Recently, my coworkers, Mr. Ralph Young and Mr. George Grubbs, and I have observed that tumor development may also be influenced by the administration of repeated large doses of homologous virus given some time after the initial infecting dose but before the appearance of a tumor (12, 13).

The first such experiment was carried out with SV-40. Each of one litter of newborn hamsters was infected subcutaneously with 0.2 ml. of an SV-40 preparation containing 10^{7.5} TCID₅₀ per 0.2 ml. Twenty-seven days later the litter was divided and half of the animals were set aside as controls. Each of the remaining hamsters was injected subcutaneously with 0.5 ml. of undiluted SV-40, and the injections were repeated at twice weekly intervals, usually on Mondays and Fridays, until 13 doses had been given. On the

91st day, one of the control hamsters had a tumor and by the 161st day all six of the control hamsters had tumors; they were all dead by the 215th day. At this time, all the hamsters which had been given multiple doses of virus were alive and well. One animal did develop a tumor on the 259th day. No tumors occurred in the remaining hamsters; three died after 516 to 539 days, and the last two were killed after 541 days.

Similar experiments were carried out with newborn hamsters infected with adenovirus type 12, except that the additional homologous virus injections were started sooner because the incubation time for adenovirus type 12-induced tumors is less than for SV-40. Similar results were obtained. Fewer tumors developed in hamsters which had received multiple doses of virus than in the hamsters which had received only one dose when newborn.

Additional doses of homologous virus injected into a hamster with either a large or small virus-induced tumor had no effect on the continued development of the tumor or on the animal's survival time.

The time for starting the first additional dose of virus to the neonatally infected hamsters was arbitrary, so different times were tried. Variations in the results were noted when additional virus doses were administered at different times following the initial infection but prior to tumor development, but this varied from experiment to experiment as is shown in table V. It

was concluded that although time was an important factor, it was not the only one.

The next likely factor considered was the dose of virus used to infect the newborn hamster. This proved to be important and the number of tumors arising in hamsters given multiple doses of virus varied with the concentration of the initial infecting dose. Table VI shows the influence of the initial infecting dose of SV-40. Results were similar when hamsters were initially infected with different dilutions of adenovirus type 12.

Whereas more tumors developed in hamsters injected with large initial infecting doses of virus, fewer tumors developed as the size of the additional virus doses was increased. Three identical groups of hamsters were infected when newborn with SV-40. In the control group of 13 hamsters, all developed tumors; in the second group of 12 hamsters which had been given thirteen 0.5-ml. doses of virus, 2 developed tumors; while in the third group of 8 hamsters which received thirteen 1-ml. doses of virus, all remained free of tumors. Likewise in three groups of hamsters infected neonatally with adenovirus type 12; 12 of 14 hamsters which received no other virus developed tumors; in a group given 13 additional one-quarter ml. doses, 6 of 13 developed tumors; while only 2 of 14 hamsters in a group given 13 additional 1-ml. doses developed tumors.

TABLE V.—Influence of Time of Administration of Large Doses of SV-40 to Hamsters Infected With the Virus When Newborn on Tumor Development

Hamsters infected with SV-40 when newborn						
Controls	Additional doses of virus started on days:					Observation period (days)
	8	15-16	25-26	38-46	60-94	
7/7		1 1/7	0/7	0/7	3/7	371-447
5/6				5/6		375
5/6				6/6		301-509
5/6					5/6	245
8/8				6/7		383
7/7			0/7			228-378
9/11			3/11			343
7/7			4/7			307
5/7			0/6			307
9/9			3/8			223
12/13		3/13				258
10/10	7/19					
Total 89/97	7/19	4/20	10/46	17/26	8/13	
Percent 90	37	20	22	65	62	

¹ The denominator denotes the number of hamsters injected and the numerator the number of hamsters which developed tumors.

TABLE VI.—Influence of Initial Infecting Dose of SV-40 Given to Newborn Hamsters on the Protection From Oncogenicity Resulting From the Administration of Additional Large Doses of Undiluted Homologous Virus

Observation period (days)	Dilution of initial infecting virus							
	Undiluted		1:4		1:16		1:64	
	control ¹	treated ¹	control	treated	control	treated	control	treated
319-314	² 7/7	4/7	6/6	2/6	2/2	0/2	5/7	0/6
214-328	8/8	2/7	3/4	1/5			6/7	0/7
Total	15/15	6/14	9/10	3/11	2/2	0/2	11/14	0/13
Percent	100	43	90	27	100	0	79	0

¹ The "control" hamsters received a single dose of virus when newborn; the "treated" hamsters received additional repeated large doses of virus beginning 25 days after the initial infecting dose of virus.

² The denominator denotes the number of hamsters injected and the numerator the number of hamsters which developed tumors.

To determine if the large doses of virus administered to the already infected hamsters could exert their effect by inhibiting viral antibody and if in some way this might have inhibited tumor formation, hamsters which had received only one infecting dose of adenovirus type 12 and hamsters which had received the initial infecting virus dose plus additional large doses of virus were bled and their sera were tested for neutralizing antibodies in human embryonic kidney cell cultures. In each instance the hamsters which had received multiple doses of virus had higher antibody titers than the hamsters which received only one dose of virus. Eventually the tumor-bearing control hamsters also developed high titer viral antibody (table VII).

The usual number of additional virus doses given was 13. This number was varied in one experiment

TABLE VII.—Neutralizing Antibody for Adenovirus Type 12 in Sera of Hamsters Infected When Newborn, Then Untreated or Treated With Additional Doses of Homologous Virus

Untreated hamsters			
Number	Tumors	Time of bleeding	Titer
A3703	None	14 days	1:4
A3737	None	21 days	1:8
A3787	None	36 days	1:4
A3499	Yes after 75 days	109 days	>1:512
Treated hamsters			
A3704	None	14 days	1:16
A3736	None	21 days	1:16
A3788	None	36 days	1:512

with adenovirus type 12 and two with SV-40. In the hamsters infected with adenovirus type 12, all 10 control hamsters which received only the initial infecting dose of virus developed tumors. In the groups given either 1, 3, or 8 additional doses of virus, 6 of the 10 hamsters in each group developed tumors, while in the group of 8 hamsters which received 13 additional doses of virus only 1 developed a tumor. The two experiments with SV-40 are shown in table VIII. The additional doses of virus were started somewhat late. In the first test, half the animals in each group began receiving virus 30 days after the initial infection, and the other half, 37 days after the initial infection. The inhibition of tumor development even with 13 additional doses of virus was less than usual. In the second test the virus preparation used induced tumors in only five of seven control hamsters, and even one additional dose of virus had an inhibitory effect.

In each of the experiments described, homologous virus was administered after the initial infecting dose. To test the type specificity of the inhibition of tumors by repeated injections of adenovirus-induced tumors, three groups of hamsters were infected when newborn with adenovirus type 12. Group 1 was kept as controls, Group 2 was given 13 additional doses of adenovirus type 12, and Group 3 received 13 additional doses of adenovirus type 7. Tumors developed in some of the animals in Group 3, the group which received additional doses of adenovirus type 7, but the numbers of tumor-bearing hamsters were less than in Group 1, the control group, and more than in Group 2, the hamsters which received additional doses of the homologous virus. The experiment was repeated twice with similar results as is shown in table IX. The dif-

TABLE VIII.—Influence of the Number of Doses of Homologous Virus Administered to Hamsters Infected With SV-40 When Newborn on the Development of Tumors

Additional doses of virus started on day:	Observation period (days)	Hamsters infected with SV-40 when newborn				
		Controls	Treated with additional doses of SV-40			
			1X	3X	8X	13X
30-37	287-434	¹ 12/12	10/11	7/11	4/10	6/11
29	362-442	5/7	0/7	1/7	1/8	0/8
Total		17/19	10/18	8/18	5/18	6/19
Percent		89	55	44	28	32

¹ The denominator denotes the number of hamsters injected and the numerator the number of hamsters which developed tumors.

ference is not impressive and the numbers are still small so their significance is uncertain.

The nature of the inhibitory effect of additional doses of virus on tumor development is uncertain at present. A number of preliminary experiments designed to determine the mechanism of the inhibition have been carried out or are under way, but to date conclusive results are lacking.

The inhibition of tumor development following repeated doses of homologous virus suggested that a vaccine might be useful in the prevention of tumors. It was not clear whether the protection depended on the initial infection, nor was it clear whether repeated injections of large doses of an oncogenic virus to a normal animal would induce tumors. Accordingly, repeated large doses of adenovirus type 12 were injected into 4 litters of normal hamsters starting when

11 to 15 days of age. The animals were kept for over 200 days and not a single one developed a tumor, indicating that the inhibitory effect on tumor induction did not depend on the initial infecting dose of virus given when newborn.

That repeated doses of the same oncogenic virus in hamsters results in fewer tumors than when only one dose of virus is given invites speculation on the effect of all the improved sanitary measures that have been adopted by man and which must serve to prevent repeated infections with many disease-producing microorganisms. Poliomyelitis was observed to become a severer disease and to affect older people after improved sanitary measures were instituted. In the event that viruses cause tumors in man, there is the possibility that they may have become more oncogenic as the chances for repeated infections were reduced.

TABLE IX.—Effect on Tumor Induction in Hamsters Infected With Adenovirus Type 12 When Newborn, Then Given Repeated Large Doses of Adenovirus Type 12 or Adenovirus Type 7 Beginning 12 to 15 Days After the Initial Infection

Hamsters infected with adenovirus													
One dose of type 12 when newborn only					Additional doses of type 12					Additional doses of type 7			
Tumors	Per-cent	Time (days)		Extra virus injections started on day:	Tumors	Per-cent	Time (days)		Extra virus injections started on day:	Tumors	Per-cent	Time (days)	
		Tumor development	Observation period				Tumor development	Observation period				Tumor development	Observation period
¹ 6/7	86	34-42	181	15	4/9	44	34-83	181	15	6/8	75	34-69	181
10/13	77	28-65	126	12	2/12	17	35-49	126	12	5/11	45	35-56	126
9/12	75	32-77	133	13	1/13	8	86	133	13	7/12	58	35-77	133
Total	25/32	78			7/34	21				18/31	58		

¹ The denominator indicates the number of hamsters injected and the numerator the number of hamsters which developed tumors.

SUMMARY

Cell cultures infected with either SV-40 or adenovirus type 12 may react differently. Infectious virus may be released by the cells with or without accompanying cell destruction, or the infective virus may transform the cells to abnormal, wildly growing cells, then disappear. Cells which release infectious virus without being destroyed and the transformed cells which are free of infectious virus may be kept for a long period of time in vitro. There is no explanation at present as to why some cell cultures continue to yield infectious virus while others become transformed and do not yield infectious virus.

Certain factors which determine whether SV-40 or adenovirus type 12 will induce tumors in hamsters are known: these are age, species of virus, concentration of virus used to infect the newborn hamster, and whether the animals received additional doses of the homologous virus some time after the initial infecting virus dose but before tumors appear. Susceptibility decreases with age and older hamsters are not susceptible to tumor induction either with SV-40 or adenovirus type 12. In hamsters, SV-40-induced tumors take three or four times longer to develop than adenovirus type 12 tumors. High concentration of virus on injection into newborn hamsters will induce tumors in every animal or almost every animal and the incubation time for tumor development and death of the animal is decreased as the concentration is increased. Repeated injections of homologous virus some time after the initial infecting dose of virus but prior to the appearance of tumors has an inhibitory effect on tumor development. The extent of the inhibition depends largely on the concentration of the initial infecting dose of virus and to a lesser extent on the size and number of the additional virus doses given. In general, maximum inhibition is obtained when large doses of homologous virus are administered to the infected animals and when such doses of virus are repeated many times.

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Persistent Viral Infection Without Production of Infectious Virus

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Attempts to incriminate viral infection as a cause of chronic disease processes have generally been predicated on the same basic assumption that underlies the testing of hypotheses on viral etiology of acute disease; that is, that the virus is present, in an infectious state, in the diseased tissue. In the field of tumor virology this assumption has undergone intensive scrutiny within recent years, and some of the lines of thought in this area may be relevant to other chronic diseases.

First, it must be recognized that the above assumption can be divided into two portions: (1) that the virus is present, and (2) that it is in an infectious form. A major achievement of tumor virology has been to show that in virus-induced tumors, the first is almost always true, but the second is frequently not. This understanding has come about in large part from our increasing insight into the nature of viruses and their multiplication, first in bacteriophage systems, and more recently in animal virus systems. In essence, these studies have shown that viruses are nucleic acids which are enclosed in a protective capsid or envelope and which utilize host cell mechanisms for carrying out a complex process involving synthesis of new enzymes, viral structural proteins, and viral nucleic acid, and combining the separately synthesized components into new infectious particles. Thus, formation of infectious virus by a cell is only the end stage of a complex and delicately synchronized process which can proceed with greater or lesser efficiency and which can be disrupted at many points. We cannot use production of virus as the criterion of whether a cell is infected, but must think of an infected cell as one in which viral genetic material (perhaps only a portion of the viral genome) is present in a stable and potentially functional form. An infected cell is simply

a cell with a new piece of nucleic acid (viral in origin).

There are now a number of instances in tumor virology of chronic or persistent viral infection without production of infectious virus; in some systems the whole viral genome is present, while in others it appears that only certain genes are present. Persistence of the complete genome is exemplified by certain hamster tumors induced by SV-40 virus. These tumor cell lines, generally carried in tissue culture, are devoid of infectious virus; however, if the cells are grown in direct contact with susceptible monkey cells or transplanted into hamsters, virus often can be recovered; (1-3). Also, treatment of the tumor cell cultures with various chemicals occasionally results in production of a few infectious virus particles (4, 5).

There are several examples of persistence of only a portion of the viral genome, both with RNA and DNA viruses. Certain chicken cells transformed by Rous sarcoma virus produce no infectious virus, but the viral gene responsible for inducing transformation can be rescued from such cells by superinfection with related avian leukosis viruses. The progeny of such infection contain particles with the gene for transformation but with the antigenic characteristics of the superinfecting virus (6). Also, as described below, the transformed, non-virus-producing cells are producing one of the antigens of the avian leukosis virus group.

Persistence of genes of DNA and RNA viruses in tumor cells has been shown most frequently by the continued production of viral antigens in the absence of viral infectivity detectable by any direct or indirect procedure. By viral antigens is meant antigens coded for by viral nucleic acid, whether or not the antigens actually are incorporated into the infectious virus particles. Several classes of antigens have been observed.

One class is recognized by sensitive tumor transplant rejection tests, previously infected animals having some ability to restrict growth of transplants of tumor cells induced by the homologous virus (7-14). These transplantation antigens are apparently present in the cell membrane, and have been observed with both DNA and RNA tumor viruses. A second class of antigen is structural components of the virus particles; these are formed in tumors induced by avian leukosis (15) and mouse leukemia viruses (16) and in small amounts in adenovirus tumors (17).

The third class, which is probably of greatest relevance to the problem of chronic viral disease, is the recently recognized group of antigens known as T antigens or neoantigens (18-25). These antigens, at present known only for some DNA viruses, are proteins formed early in the latent period of infection and apparently not incorporated into the virus particle. They are found in abundance in tumor cells even though structural antigens and infectious virus are not present. They are generally unstable and poorly antigenic; probably because of these properties the only source of antibody to neoantigens is the serum of animals which have carried the virus-induced tumors for prolonged periods. The neoantigens can be detected by various in vitro serological procedures, namely, complement fixation, immunofluorescence (26-29) and immunodiffusion (30), using tumored hamster serum tested against either tumor cells or acutely infected cells. In the fluorescent antibody preparations it is seen that essentially every tumor cell contains the antigen; the SV-40 neoantigen is seen as homogeneous or slightly granular material filling the nucleus while the adenovirus neoantigen (or, more likely, neoantigens) is found in both nucleus and cytoplasm, in the form of fine flecks. Tumor cells continue to produce these antigens indefinitely throughout long series of passages in animals or tissue culture.

The possible importance of these findings for chronic disease studies lies chiefly in the possibilities they may offer for retrospective diagnosis. It is entirely conceivable that a certain proportion of cells infected long before with a DNA virus may, like tumor cells, harbor a portion of the viral nucleic acid and chronically produce the neoantigen of that virus. Persistent neoantigen production could be not only a marker of past viral infection, but could also play a role in the pathogenesis of chronic illness; possible mechanisms include disturbance of cellular function or initiation of autoimmune responses.

Formidable technical difficulties must be overcome before these diagnostic potentialities can be tested. While it is probable that all DNA viruses induce neoantigens, and RNA viruses conceivably could also, a means must be found for obtaining antibody to the neoantigens of nontumorigenic viruses before these concepts can be widely applied.

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DISCUSSION

GAJDUSEK: If you have a tissue culture which you presume is producing T antigens, but which you cannot use as an antigen to get anti-T antibody, could you implant it so that it grows in an animal as a transplanted tumor?

ROWE: We could possibly infect a hamster tumor in tissue culture and then put it back into hamsters.

GAJDUSEK: What about HeLa cells or other established cell lines? Can you get these to grow in a laboratory animal?

ROWE: The problem there is heterologous species reaction. You would get so much antihuman antibody that you could not filter out what was significant. The nice thing about our system is that it is in the same hamster, at least in the case of primary tumors.

BANG: If you were to attempt to apply this to chronic disease in man, shall we say, of some kind that is nontumorous, you would set up tissue cultures of this particular diseased area and then test for the possibility of serum taken from a diseased person doing something to this particular group of cells, thereby hoping to produce evidence of some specific entity. However, if the test was positive one would have to rule out autoantibodies.

ROWE: We run into that in complement-fixation tests.

BANG: Have you seen any way out of this?

ROWE: Not really; not without a far better understanding of the way it works.

BANG: You have managed to avoid this because you have several different viruses producing tumors, and an antiserum that is specific for an adenovirus tumor antigen is different from an antiserum for SV-40 tumor virus, but you have to have several agents producing the same disease in order to get this differentiation.

ROWE: We could set up the hypothesis, for example, that certain adenoviruses produce a chronic disease—chronic tonsillitis would be a good hypothesis—and we could grow these cells in culture and

stain them with hamster antiserum. This would stain the neoantigens, and in the early period in culture would give an indication of how many cells are infected. Fortunately, with the adenoviruses, the hamster serum will stain essentially all the human adenoviruses, so one reagent would suffice for these tests.

GAJDUSEK: Dr. Rowe, would this be a possible experiment to test your hypothesis that a recovered animal from an acute viral infection might still be bearing the genetic material for T antigen production: take identical twins of any animal, infect one with one of your acute viruses, then take from the recovered twin thymus or spleen or any cell that you think has been infected, and plant it into the identical twin and thus get from that animal antisera that would demonstrate your T antigen?

ROWE: I think that this is thinking in the right direction, if you can get a system in which enough cells are infected.

Q: Dr. Rowe, you mentioned that these early proteins are probably enzymes. How do you know that they are enzymes?

ROWE: I do not know; this is by analogy with herpes and vaccinia. It could, I suppose, be an internal structural protein, but the point is that it is formed early. It is formed 8 hours before the external ma-

terial, which fits in with it being a functional protein.

Q: What about an uncoating protein?

ROWE: As I understand it, an uncoating protein has to be surface protein; the early protein is clearly not a surface protein in that antibody against it has no effect on the virus infectivity.

POSKANZER: In order to make this a generally useful system, you would have to find virus particles that will produce tumors for every type of virus. Is there any way this could be done?

ROWE: I suspect that a physical chemist could concentrate, purify, and stabilize neoantigen of nontumorigenic viruses, and one could then immunize with large doses.

GAJDUSEK: Is there any way that you could get recombination from partially inactivated material with cells that contain the T tumor antigens, but perhaps not the rest of the replicating virus, by using partially inactivated virus?

ROWE: There have been a few attempts at this, by nucleic acid hybridization.

FIELD: Is there any way of making this virus produce a simple tumor, rather than an invasive malignant one?

ROWE: Actually most of these are really benign tumors. The rate of metastasis is very low and they are not highly invasive.

Genetics in the Pathogenesis of Subacute and Chronic Virus Infections

Chairman

K. HABEL

Genetic and Cellular Factors in Resistance to Virus Diseases

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It has long been recognized that individuals vary greatly in susceptibility to diseases. In the various experimental infections of animals, once it was established that disease susceptibility would vary from one stock of animals to another, the way was open to select certain stocks for resistance or susceptibility to particular bacterial or viral agents. This was true of plants as well. Haldane (1) was one of the first to recognize that variation in disease susceptibility has been, and is, a selective factor in the evolution of all plants and all animals, including man.

This report will review some of the factors which are important in the resistance of mice to the virus of mouse hepatitis, which we have studied in some detail, and also refer to some of the work which has been done on the resistance of mice to one or two arboviruses. Webster, who initiated experimental epidemiology in America, was among the first to recognize the significance of genetic variation. He showed that selected strains of mice were resistant to certain bacteria and susceptible to certain viruses, while other selected strains were resistant to viruses and susceptible to bacteria. Resistance therefore was not a general capacity possessed by certain animals, but was referable to particular organisms: "resistant" animals were resistant to certain organisms but fully susceptible to others.

About 13 years ago, the first clear study of the genetic susceptibility of an animal to a virus disease was done by Sabin (2) (fig. 1). He compared two strains of mice, the PRI strain and the Swiss strain. Swiss mice were invariably susceptible, and Princeton mice invariably resistant to the 17 D strain of yellow fever virus, which is used as a vaccine for man. Not only were the Princeton mice 100 percent resistant (that is, they all survived) and the Swiss mice 100 percent

susceptible; but standard genetic studies showed a series of ratios which clearly indicated that this genetic susceptibility and resistance was a unifactorial characteristic. This was especially important since it suggested an inherited resistance to viruses affecting the central nervous system. Sabin, however, was very much aware that a number of factors might influence this clear-cut demonstration of susceptibility and its genetic nature. First, he pointed out that if instead of the 17 D strain one used the French neurotropic strain of yellow fever virus, a proportion of the adult PRI resistant mice were killed. He showed that although other members of the arbovirus group B (such as dengue, West Nile, Japanese B, St. Louis, and Russian spring-summer viruses) did not grow in the resistant mice, other neurotropic viruses, particularly members of the arbovirus group A (such as western, eastern, and Venezuelan equine encephalitis) and other viruses which attack the central nervous system (poliomyelitis, rabies, lymphocytic chorio-meningitis, Rift Valley fever, etc.), were not inhibited. Therefore, the genetic resistance of the mice was not a general resistance but was very specific. He also pointed to another variation in susceptibility, which is not genetic but which will be recurring in our discussion. This is that very young mice from the resistant strain are just as susceptible to the more virulent strain of virus as are adults of the susceptible strains.

We stumbled on this problem about 7 years ago while attempting to grow another virus, mouse hepatitis, in mouse tissue cultures. This virus had been discovered by two groups of workers, Gledhill and Andrewes (3) in England, and Nelson (4) in the United States, independently of each other; and they had emphasized two different factors. Nelson discovered the virus when he was passing the cells of a lymphoma

MECHANISM OF INHERITANCE OF RESISTANCE TO 17D YELLOW FEVER IN MICE

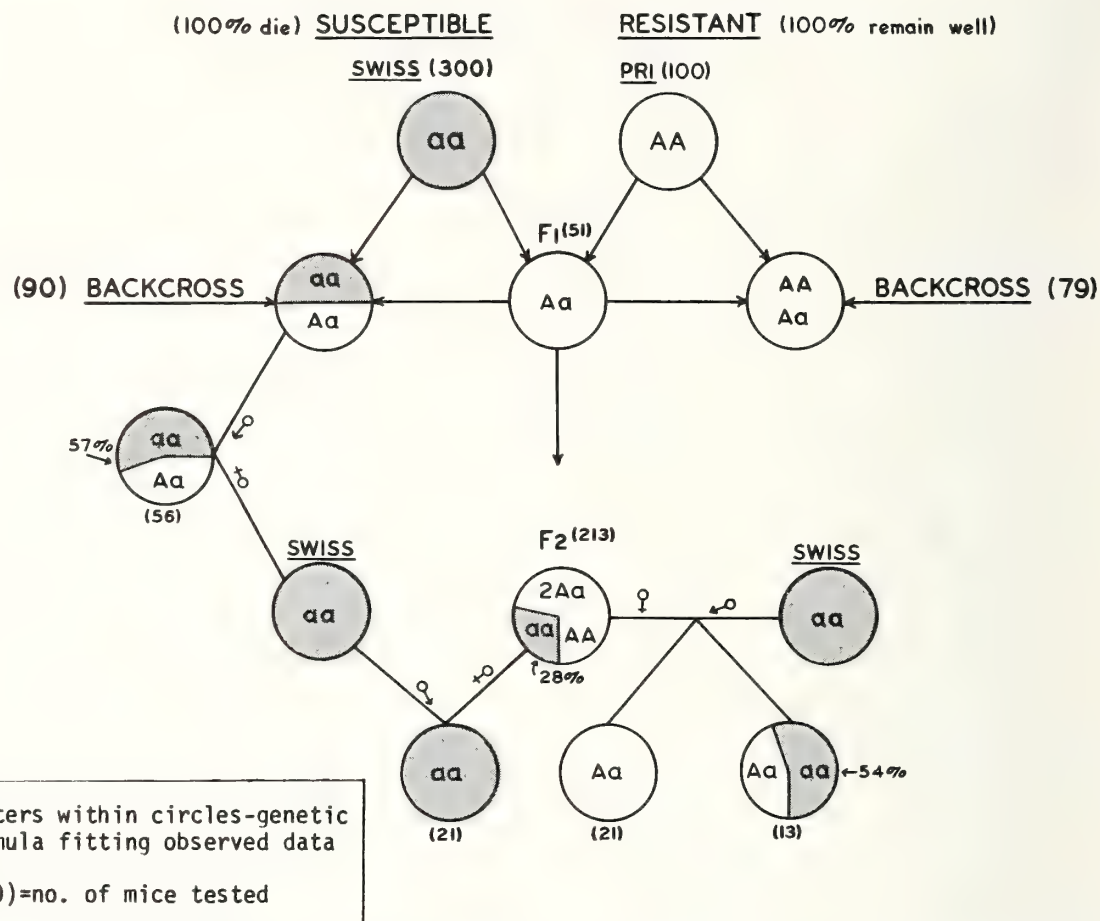


FIGURE 1.—Mechanism of inheritance of resistance to 17 D yellow fever virus in mice. (Courtesy of Dr. Sabin.)

from mouse to mouse in the acute stage. During the continued passage of this material, a number of his mice came down with hepatitis; and he found that this associated hepatitis was due to a separate agent, which had presumably been picked up by the lymphoma cells during their passage. Gledhill and Andrewes (3) had shown that their clinical mouse hepatitis was dependent not only upon the virus itself but upon an associated small organism called eperythrozoon, which grew on or in red cells and which somehow enabled the mouse to become susceptible to the virus.

While trying to grow this virus in tissue culture, our attention was drawn to macrophages by the observation that the virus seemed to affect Kupffer cells in the intact mouse. When bits of mouse liver are

placed on collagen, the macrophages present in the liver grow and spread out from the liver, but migrate away from the collagen material, and are found on the opposite side of the culture tube. When we turned the tubes around and looked at our infected cultures, it was immediately apparent that the virus had selectively destroyed the macrophages and spared the fibroblasts and the epithelial cells, or the parenchymal cells, of the liver tissue. The virus was then carried through a series of passages in mouse liver cultures, and it was clear that the virus indeed did multiply rapidly; that during the course of its multiplication, it destroyed the macrophages at every passage; that it grew to a relatively high titer; and that after a number of passages in tissue culture, it acquired also the

capacity to destroy some of the liver parenchymal cells. This selective capacity of the virus to grow and to destroy macrophages is in itself a fascinating problem.

Let me turn, however, to a study of the genetic aspects of this macrophage susceptibility. Since it was evident that the macrophages were highly susceptible, the question arose whether this was a factor inherent in the host cells, or whether cells from other hosts might be found susceptible. Instead of turning to other species, we first turned to another strain of mice; and since a C₃H mouse strain was commonly kept in the laboratory, we tested this for susceptibility. To our surprise, we found that C₃H mouse liver cultures, set up in exactly the same way as the PRI cultures, were resistant: they did not show destruction of the cells, did not yield new virus, and all of the cells appeared perfectly normal, even when inoculated with relatively large doses of virus.

Knowing of Sabin's work on the genetically based variation in susceptibility, the first question, of course, was whether the macrophages obtained from susceptible mice were susceptible because they reflected a genetic factor. Studies on genetic susceptibility were done first on the mice themselves, and it was relatively easy to demonstrate that there was a genetic susceptibility of the Princeton mice to the particular strain of virus which we had obtained from Dr. Nelson, and that this factor was Mendelian in nature (fig. 2).

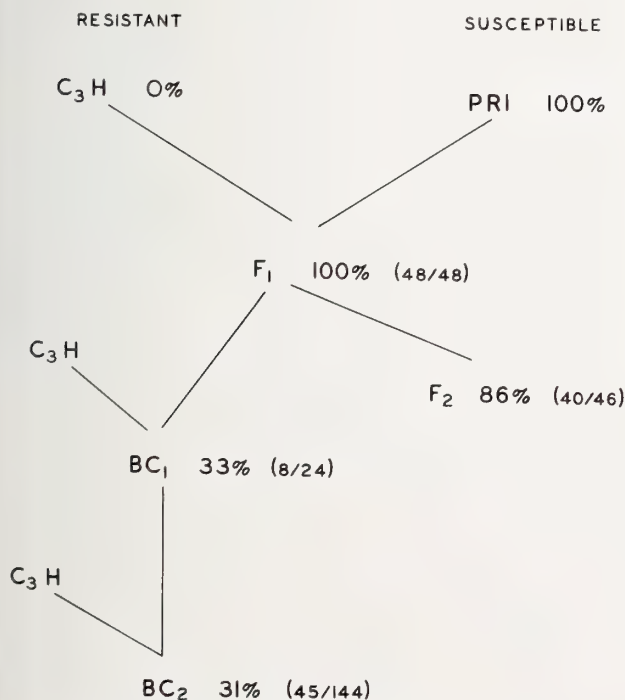


FIGURE 2.—Test for mouse susceptibility to MHV.

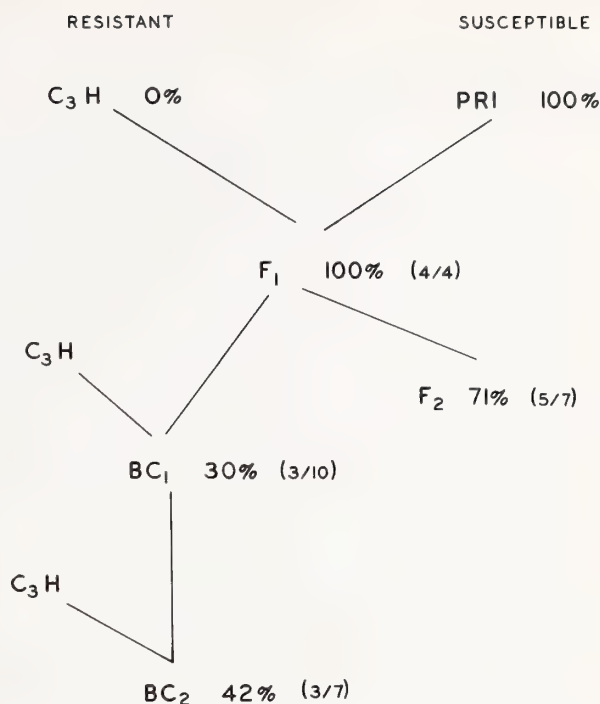


FIGURE 3.—Test for liver macrophage susceptibility to MHV.

The next question was whether the macrophages represented this genetic susceptibility. This was tested by taking the baby mice from various crosses and back-crosses and setting up cultures of macrophages from individual mice. About six cultures were set up, half of which were tested for susceptibility and half kept as controls. The ratios, shown in figure 3, represent individual mice, that is, several cultures from one individual mouse were tested for susceptibility or resistance; and if it was recorded susceptible, it meant that all of the cultures from that particular mouse were demonstrated to be susceptible, and if recorded resistant, that all of the cultures from that particular mouse were resistant. The chart shows that the same crossing, segregation, and back-crossing results were obtained on cultures of macrophages as were obtained on weanling mice themselves. Thus, there was a close correlation between the ratios, and it was concluded that the macrophages themselves, acting as the host cells for mouse hepatitis, represent the phenotypic expression of the genetic variation in susceptibility to this agent.

We have followed this trail somewhat further, and as a result there are several reasons why we believe that macrophages are the basic cells involved in susceptibility to this agent. First, there are the data which have just been outlined. Secondly, macrophages obtained from other areas such as the lung and from peritoneal

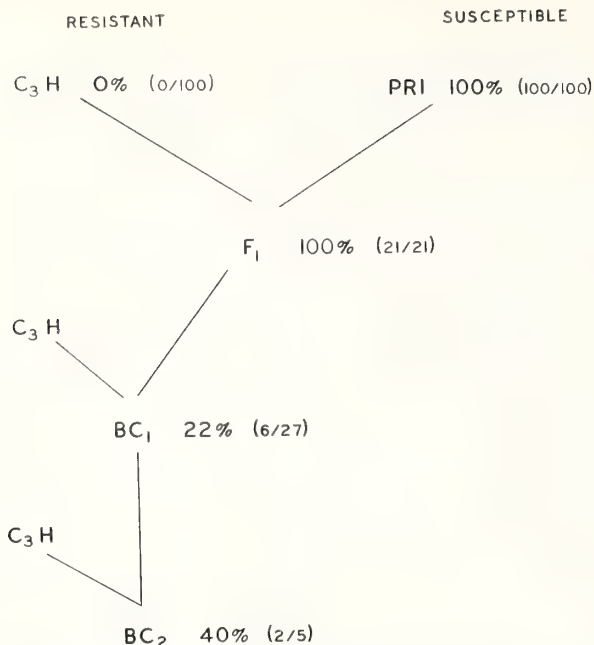


FIGURE 4—Test for peritoneal macrophage susceptibility to MHV.

washings are susceptible if they are obtained from the susceptible strain of mice, and resistant if obtained from the resistant strain of mice (fig. 4). It is clear from the back-crosses now completed that one genetic effect is also unifactorial, for about 50 percent of the back-crosses are susceptible (table I). Moreover, the resistant and the susceptible back-crosses differ markedly in susceptibility. The resistant strain shows destruction only when inoculated with about 10^6 infectious doses, while one millionth of this caused the susceptible back-cross cells to be destroyed (figs. 5, 6, 7). Third, in a small series of mice in which we were able to test susceptibility or resistance by four different

TABLE I.—The Susceptibility of Back-Cross Generations (Number Susceptible/Total Number Tested)

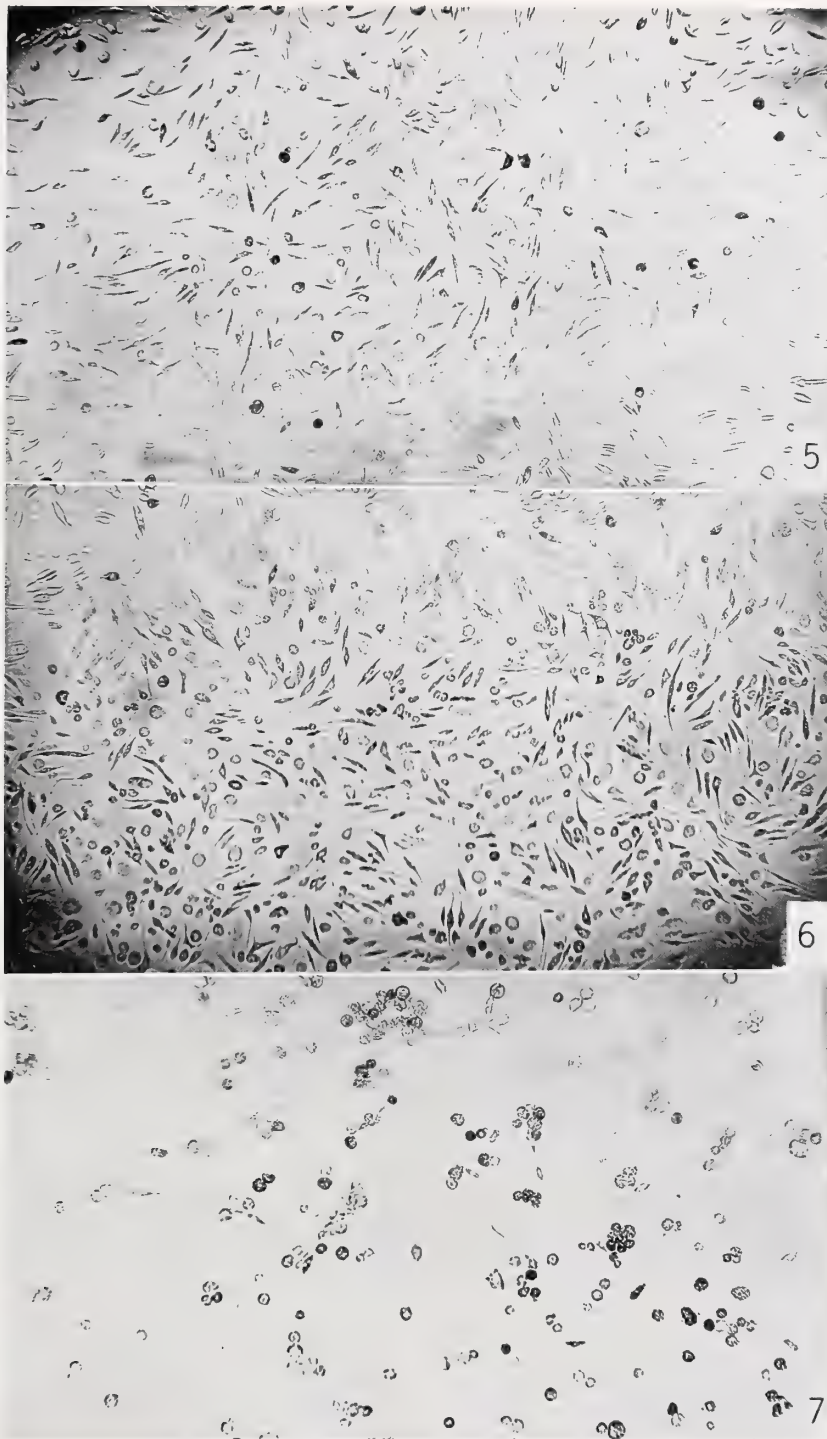
Mice	Peritoneal macrophage cultures	Percent susceptible
BC ₁	6/27	22
BC ₂	2/5	40
BC ₃	10/41	47
BC ₄	3/7	42
BC ₅	10/31	32
BC ₆	15/30	50
BC ₇	14/30	47
BC ₈	4/8	50

ways, there was a very good correlation between the four methods of testing. Table II shows the results of these studies and demonstrates the close correlation. In one case, there is an exception; but if this particular mouse had been tested more fully, a few susceptible descendants would probably have been found.

Fourthly, additional evidence that there is a correlation between the events in the cells and the events in the intact host mice is found in the story of the young mice. As mentioned earlier, Sabin had pointed out that resistance readily demonstrable in adult mice may not be so clearly present in baby mice of the same strain. It is well recognized that while a young animal may be susceptible to a given disease, adults of the same species may have some degree of resistance to the same disease even if they have not been specifically exposed to it. This turned out to be true of our pair of susceptible and resistant mice. If baby mice of the resistant C₃H strain of mice are tested for susceptibility, they are intermediate in their susceptibility. By this I mean that they do not die within two or three days as

TABLE II.—Susceptibility of Individual Mice to MHV by Genotypic and Phenotypic Tests

	Parents		Phenotype peritoneal macrophage cultures	Genotype direct test of offspring	Genotype macrophages from newborn liver cultured from offspring	Genotype peritoneal macrophages cultured from offspring
	Female	Male				
BC ₁ female 1	F ₁	C ₃ H		4/13		2/5
BC ₁ male 2	F ₁	C ₃ H	Susceptible	5/13		
BC ₁ male 3	F ₁	C ₃ H	Susceptible	16/26		
BC ₁ male 7	F ₁	C ₃ H	Susceptible	7/12		
BC ₂ male 9	BC ₁	C ₃ H	Resistant	0/13	0/4	0/10
BC ₂ male 10	BC ₁	C ₃ H	Resistant	0/4		
BC ₂ male 13	BC ₁	C ₃ H	Susceptible	11/26	0/6	
BC ₂ female 9	BC ₁	C ₃ H	Susceptible	7/13	1/2	
BC ₂ female 10	BC ₁	C ₃ H	Resistant	0/16	0/8	



FIGURES 5-7.—Macrophages from peritoneal washings of 3 individual mice from BC_3 generation progeny of susceptible back-crossed parents. $\times 50$

- (5) 10-day-old culture, noninfected.
- (6) 10-day-old culture, 6 days after infection. Resistant.
- (7) 10-day-old culture, 6 days after infection. Susceptible.

the fully susceptible adult PRI mice do, but they die after a period of 6 or 8 days. Virus obtained from their livers and passed to other baby mice produced a rather delayed susceptibility. We had previously found that macrophages obtained from these C₃H mice were resistant. When these tests were repeated, the cells were indeed resistant in the terms in which we had originally posed the test, that is, they were not destroyed within a period of about 10 days; but if the cultures were kept for longer periods of time, they, too, showed a delayed susceptibility. Thus, there is a correlation between the delayed susceptibility of the baby C₃H mice and the delayed susceptibility of the cells obtained from baby C₃H mice (5).

A group in Philadelphia, working primarily with Koprowski, has returned to Sabin's original system, and has studied susceptibility and resistance to West Nile virus, a group B arbovirus. When they first tested liver cultures or kidney cultures (particularly cultures which had epithelial cells in them) from susceptible and resistant mice, they found them to be equally susceptible. However, when the susceptibility of cultures of peritoneal macrophages from the mice was tested, it was shown that virus grew rather poorly in those from resistant mice but grew relatively well (yielding titers of 1,000 to 10,000 infectious doses) in cultures of those from susceptible mice.

Vainio (6), from the same laboratory, has reported that macrophages obtained from spleen cultures of the susceptible mice yield a hundred to a thousand times more virus than such cultures obtained from the resistant mice; that the amount of virus inside the cells is similar to that which is released from the cells; and that the susceptible macrophages yield virus for about 19 days after the original infection, some time after resistant macrophages have ceased to yield virus. Lung cultures from susceptible mice also yielded a hundred to a thousand times more virus than those from the resistant mice. (One wonders at this stage whether these lung cultures did not have relatively large numbers of macrophages present.) Brain explants from the susceptible mice also produced a higher yield of virus than those from resistant mice. On the other hand, only slightly more virus was produced by kidney cultures from susceptible mice than by kidney cultures from the resistant mice. Staining of macrophage cultures with fluorescent antibodies showed that a good proportion of the cells were fluorescent soon after plating and that the susceptible cells yielded a higher proportion of infected cells. Even in the kidney cultures the amount of virus identified by fluorescent antibody showed a greater yield in the susceptible cells

than in the resistant ones. So Vainio concludes that the gene determining resistance to arboviruses (or to this particular arbovirus) appears to affect virus multiplication in all the tissues, but that the difference is much more marked in the macrophage cultures than in the other cells. This kind of data should act as a warning against too much early enthusiasm. It is apparent that the whole story is not told. Indeed, if one considers the various ways in which genetic factors manifest themselves, one would not expect that it could be told. However, a series of general questions may be asked:

1. Are macrophages unique in their manifestation of genetic resistance?
2. Is there a relationship between virulence and the capacity to destroy macrophages?
3. Are there other ways in which genetic resistance to disease may manifest itself?

In answer to the first—the uniqueness of the macrophages—one may expect that the particular system of cells which is most important in the pathogenesis of a particular virus infection will be the cell system to predominate in resistance. Thus in an infection in which the reticuloendothelial system is primarily involved, resistance in macrophages would be particularly important. However, as Vainio showed, the central nervous system itself may manifest differences in cell susceptibility in a neurotropic virus disease. Crittenden et al. (7) have shown that the resistance of chick embryos to Rous virus is manifest in fibroblast cultures, which is not too surprising in an infection which converts cells into sarcomas.

The second question, whether virulence may be related to the capacity of a virus to destroy macrophages, may be answered in the affirmative. Roberts (8) studied the ectromelia, or mouse pox, system and followed the susceptibility of macrophages in culture. He found that the virulent strain grew much more rapidly in macrophage cultures than did the avirulent strain. There have been similar results in the growth of different strains of other viruses in macrophage cultures. A virulent strain of Newcastle disease virus is rapidly destructive, but less virulent strains fail to destroy the cells (9). The same is true of bacterial infections: a virulent strain of *Salmonella* rapidly destroys rat and guinea pig macrophage cultures, while less virulent strains do not destroy them (10).

To the question whether genetic resistance is manifest in any other way than in macrophage resistance, one may affirm that there are several. The rapidity of antibody response is one. Schell (11) studied the model system of ectromelia, a mouse pox virus which

in its pathogenesis in the mouse resembles small pox virus in man. He found that there were indeed variations in the resistance of different strains of mice, and that the C₅₇ Black strain was much more resistant than others. Analyzing this resistance, he found that the route of inoculation played a large part, that is, that the C₅₇ Black was much more resistant to the virus if inoculated in the footpad than in the peritoneal cavity. Then he found that if all strains were inoculated intraperitoneally, the variation in resistance disappeared. Further, if the C₅₇ Black was inoculated intranasally, it became more susceptible. Returning to inoculation in the footpad, Schell followed the rate at which antibodies appeared in the blood and found good correlation between the appearance of antibodies (and the loss of virus from the blood stream) and the recovery of the mice. He proposed that the difference between resistance and susceptibility in this case was related to the stimulus of the antibody system. I believe that his evidence is good, that there is a relationship between the factors.

But we all remember from our elementary biology that the phenotype may not always reflect the genotype. In other words, there are ways of modifying the genotype's effect. Sabin warned of such modification when he pointed out that the age factor in the mice, and the strain of virus, might rapidly change the expression of the genetic factors which he had studied.

In this discussion on latent and chronic diseases, one must ask: Where does the question of genetic variation in susceptibility fit? First, it is important to remember that even the most acute infections, such as eastern equine encephalitis (12) and Newcastle disease of chickens (13), may produce infections in tissue culture which persist as long as the experimenter keeps the cultures going. Secondly, within the same host there are susceptible and resistant cells—at least so they behave in tissue culture (12). Thus the parasitic virus might maintain a balance with its host, if it has found some way to avoid an antibody response. Under these circumstances resistance and susceptibility of different cell types could lead to large differences in clinical manifestation. These are, however, genetic characteristics, and selection of resistant strains is operative only in the presence of disease. Inbreeding as carried out in mice, or selective breeding as in mink, may then lead to the expression of these genetic differences.

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DISCUSSION

Q. Are these all primary macrophage cultures?

BANG: These are all primary macrophage cultures and they can be kept in culture for a couple of months without change in susceptibility.

Q. Do they change their configuration?

BANG: Well, there may be some growth. We have used two types of macrophage cultures. We have

used cultures of embryonic liver and these give rise to a large number of macrophages which seem to increase in number and populate the cultures much more heavily with time. There may well be multiplication in these. In peritoneal macrophage cultures, counts of the number of cells have not indicated any particular increase though there are some mitoses. We have not really attempted to establish a cell line. The person that has done the most work on this, I think, is Dr. Chang at the NIH. He has shown that rat leprosy will grow in such cultures.

CASALS: Do you get other types of cells in the peritoneal washing cultures that you can recognize?

BANG: Yes. We probably get a few fibroblasts. Indeed, when we put the virus in and get massive destruction of the highly susceptible cells, there are a few other cells lying around which seem to survive, but we have not attempted to see whether these are fibroblasts.

GUSTAFSON: We have a culture of buffy coat cells from swine and sheep that have continued for 7

years. We have been interested in watching the newer cultures and the tendency they have to phagocytose other cells, and we have become interested in their interactivities. I wondered if you could use this kind of a system.

BANG: I think it could be used. I think you probably know that the macrophages of the buffy coat of the swine are highly susceptible to African swine fever and, in regard to this conference, it is worth remembering that African swine fever causes a chronic viremia and that, in nature, it acts much like lymphocytic choriomeningitis.

GUSTAFSON: There are a few animals, such as the bush pig, which do have a continued viremia, but when the domestic pig is involved the disease is highly fatal.

BANG: I believe that if you infect piglets at birth a number of them will die, but you will get some that will become chronic carriers with no antibody and will keep putting out the virus.

Hereditary Leukomelanopathy (Chediak-Higashi Syndrome of Man, Mink and Cattle)

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The Chediak-Higashi syndrome is a hereditary disease of children discovered independently by Chediak in Cuba and Higashi in Japan. Affected children have pigmented hair with grey streaks. On cursory examination the hair has a powdered appearance. They have pigmented eyes but with an increased red reflex and photophobia. Thus, they do have pigment but not as much as in the normal individual: not true albinos, they are referred to as partial albinos. Furthermore, the anomaly involves abnormal granulation of all the series of leukocytes (fig. 1).

The granules are large but they have the same histochemical and staining characteristics as normal granules. However, they are functionally abnormal in that the individuals possess low resistance to bacterial infections. Affected individuals are able to manufacture complement and apparently have normal humoral antibody mechanisms, but the cellular defense is deficient. The enlarged or gigantic granules, which are said to be the lysosomes of the leukocytes, are peroxidase positive. In the primitive cells of the human bone marrow the congenitally abnormal leukocyte granules appear as inclusion bodies in the cytoplasm.

About 3 years ago in studying Aleutian mink disease we noticed that there were inclusion bodies in

the polymorphonuclear leukocytes of the circulating blood. We thought surely that we had discovered a specific lesion of Aleutian disease, but further investigation by one of us (G.A.P.) proved that it was not a lesion of Aleutian disease but was a congenital anomaly present in all homozygous recessive Aleutian mink. Furthermore, it is exactly the same by every measure that we can put to it as the Chediak-Higashi syndrome in children (fig. 2).

These granules provide us with a convenient tag for homozygous and heterozygous animals in Aleutian type mink. One particular homozygous, double recessive Aleutian type mink, *aa*, is called triple pearl and is a partial albino with pink eyes. The heterozygous, *Aa*, Aleutian type mink of this color phase is called double pearl mink and has dark green eyes (fig. 3).

None of the heterozygous animals exhibit the abnormal trait in the leukocytes nor are they especially susceptible to bacterial infections. There are 27 genes controlling coat color in mink and only one of these is the Aleutian gene. The Aleutian gene is inherited as a simple Mendelian recessive. This may be shown by simple breeding experiments. If one shines a light into the eyes of mink at night it is easy to pick out the homozygous recessive Aleutian mink by the greater red reflex in their eyes. This had been known for a long time but no one had previously related the finding to this syndrome.

If one breeds a mink known to be heterozygous (a double pearl mink which does not express the character of the Aleutian anomaly) to a homozygous recessive for the anomaly, and studies the blood of the progeny, half will have abnormal leukocytes and half

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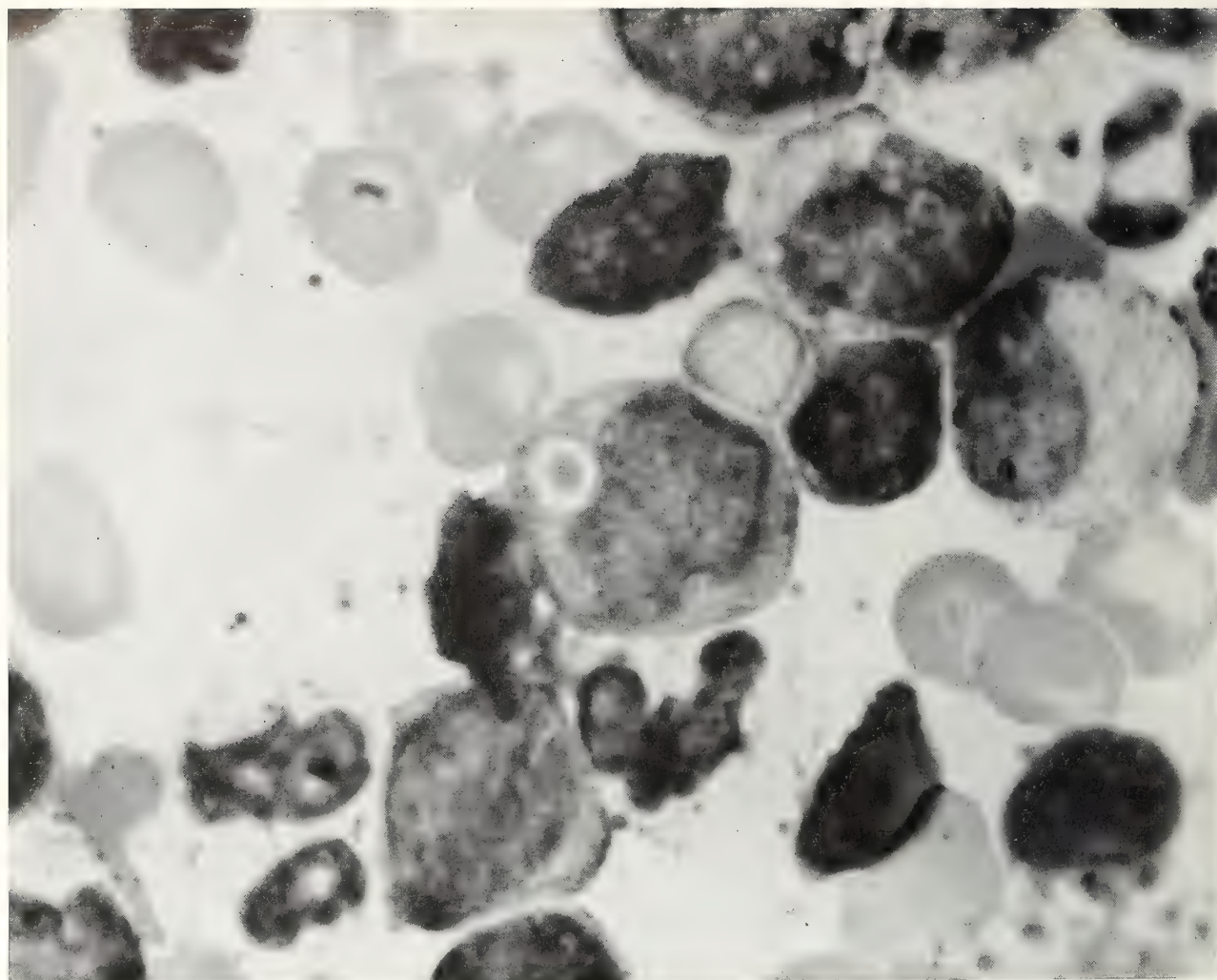


FIGURE 1.—Bone marrow impression from an affected child showing an enlarged granule in a promyelocyte. Giemsa $\times 3000$. Picture supplied by Dr. Arthur R. Page.

will not, demonstrating that the character is transmitted as a non-sex-linked simple recessive. Two homozygous recessive parents produce offspring all of which have abnormal leukocytes; and a homozygous dominant and a homozygous recessive give all carriers and none with abnormal leukocytes (table I). We have recently completed our breeding experiments by mating two heterozygotes and the expected genetic ratios resulted.

About the time these experiments were begun, one of us (G.A.P.) collected blood from every animal he could find that might be an albino and he noticed on our campus there was a herd of partial albino Hereford cattle. As far as we know it is the only such herd in the world. This was an amazing stroke of luck. When he bled these animals, the leukocytes were indeed abnormal in exactly the same fashion as

TABLE I.—The Occurrence of Leukocytic Anomaly in Mink Families of Known Matings

Parental genotype	Number of families	Number of kits	Mink with abnormal leukocytes		Mink without abnormal leukocytes	
			Male	Female	Male	Female
Aa \times aa	12	50	9	13	12	16
aa \times aa	50	240	130	110		
AA \times aa	10	42			19	23

in mink and in children (fig. 4). All of this line of cattle originated from one bull (figs. 5, 6, 7).

Instead of the characteristic dark red of a Hereford, they have buff-colored hair, less pigment in their eyes and show photophobia, squinting markedly when they

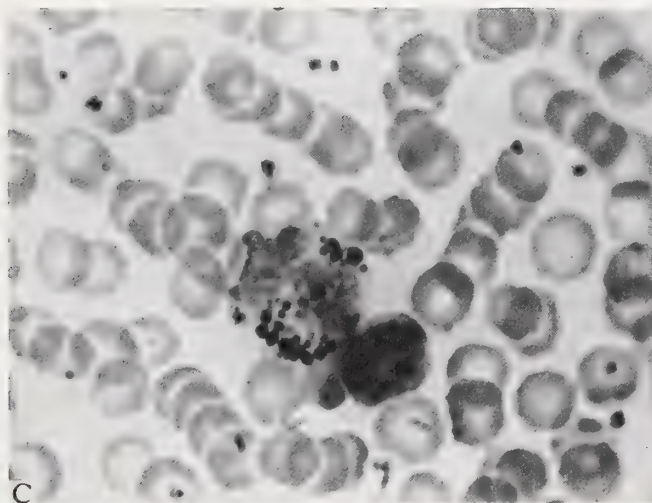
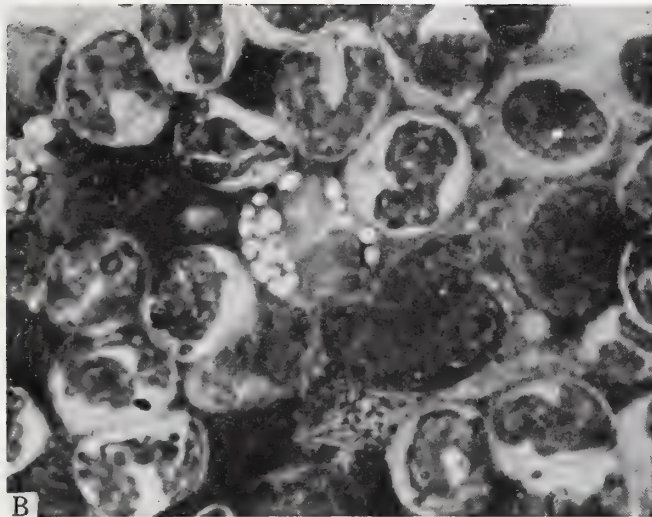
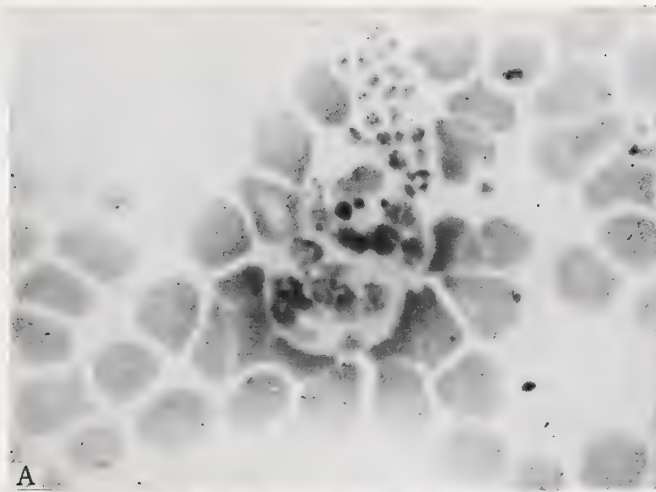


FIGURE 2.—Composite of mink cells: A. Neutrophil showing abnormal granules. Giemsa $\times 1400$. B. Myeloid cell showing abnormal granules (bone marrow impression). Giemsa $\times 1400$. C. Eosinophil showing abnormal granules. Giemsa $\times 1400$.



FIGURE 3.—Triple pearl mink *aa* and double pearl mink *Aa*. Triple pearl *aa* on the top, double pearl *Aa* on the bottom. The triple pearl is lighter in color and smaller.

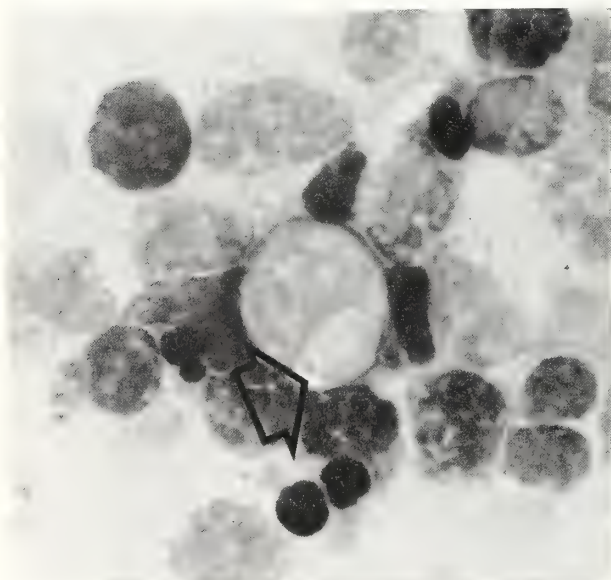


FIGURE 4.—Promyelocyte of an affected calf showing an enlarged granule. Giemsa $\times 1600$.

come out into the daylight. They are fertile and the bull will breed with a cow if she is brought into his shed, but due to the photophobia he will not mate in bright sunlight. The gene is not expressed in the heterozygote. One of our heterozygous cows was bred to a homozygous recessive bull and twinned, one calf being a heterozygous carrier and the other a homozygous recessive.

At birth the homozygous recessive is very white, but some buff colored hair develops later and darkens as the animal grows older. They are highly susceptible to bacterial infection and require very careful hospital attention. Despite this we lost one recently with a suppurative arthritis (figs. 8, 9).

If their grey eyes are sectioned the iris shows some growing melanoblasts and melanophores but in much reduced number compared to the dark eyes of normal Herefords (figs. 10, 11). Blood smears from these animals show greatly enlarged granules in the leuko-

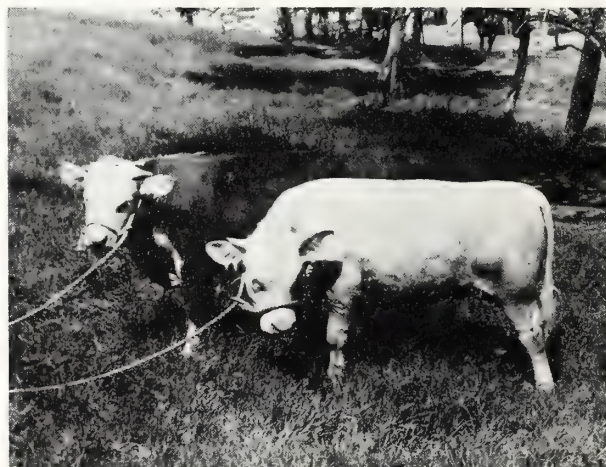


FIGURE 5.—Twin calves at 10 months of age. Partial albino in foreground.

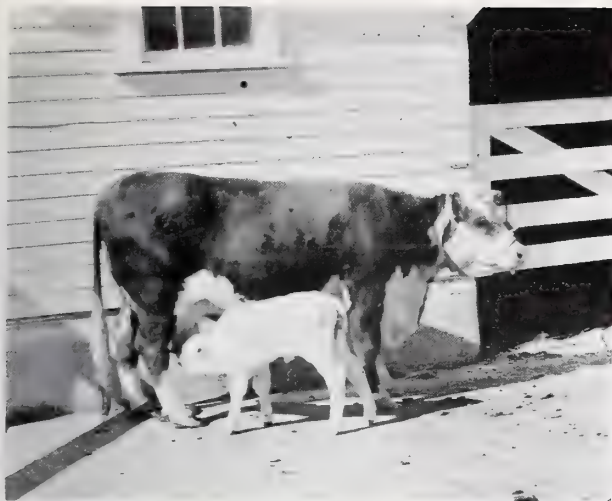


FIGURE 6.—Heterozygous cow and her homozygous calf.



FIGURE 7.—Chediak-Higashi bull. Note photophobia.

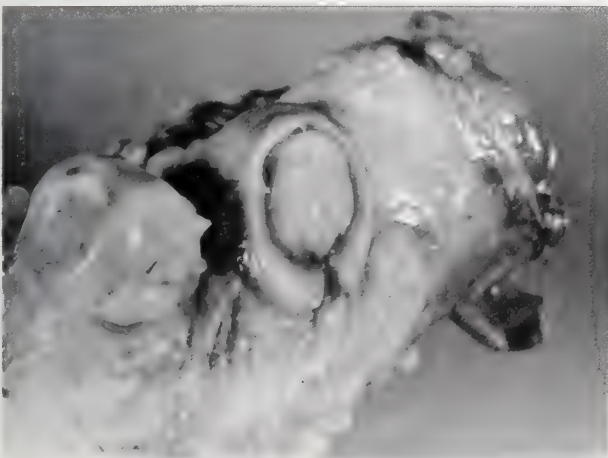


FIGURE 8.—Encapsulated joint abscess from a Chediak-Higashi calf.

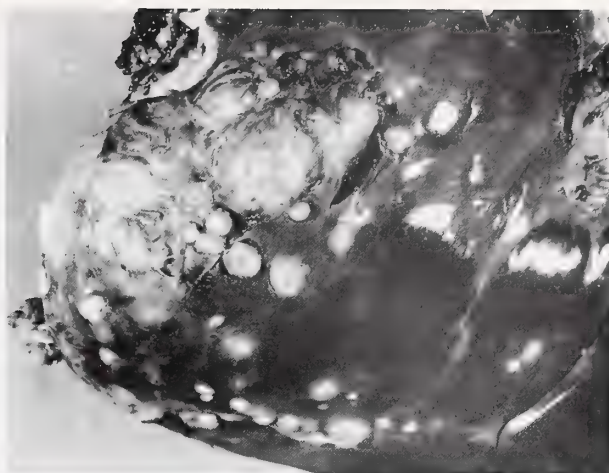


FIGURE 9.—Multiple hepatic abscesses. All Chediak-Higashi calves have had these lesions.

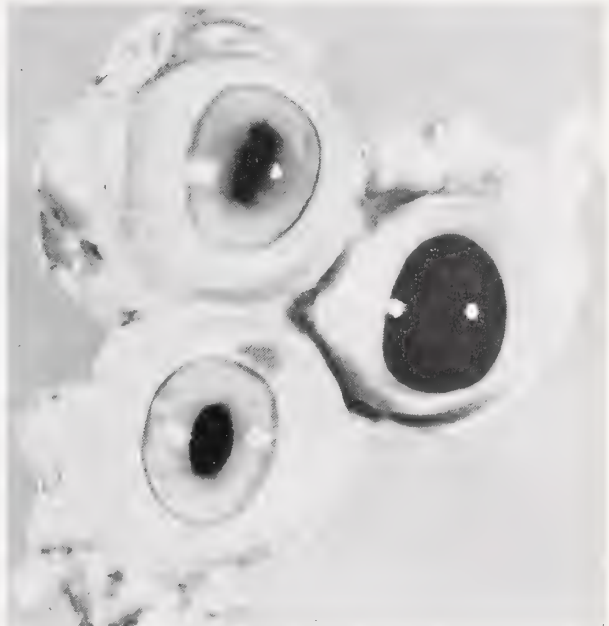
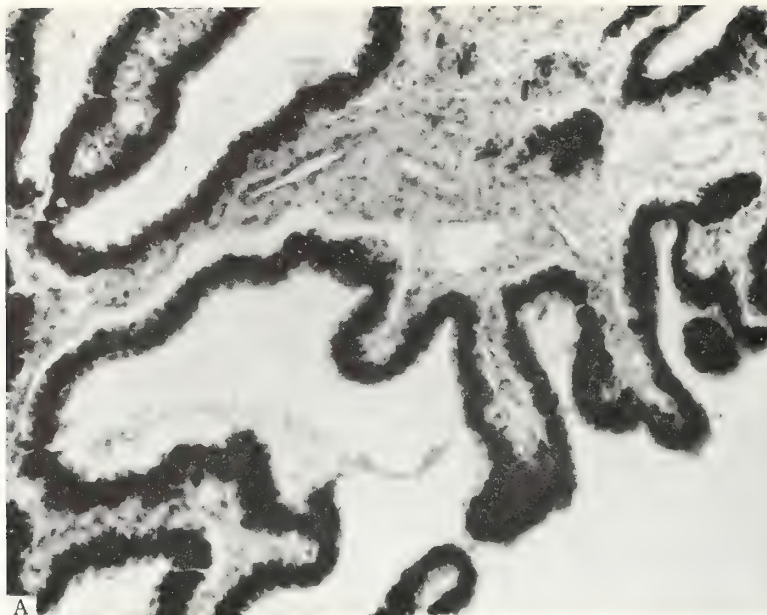


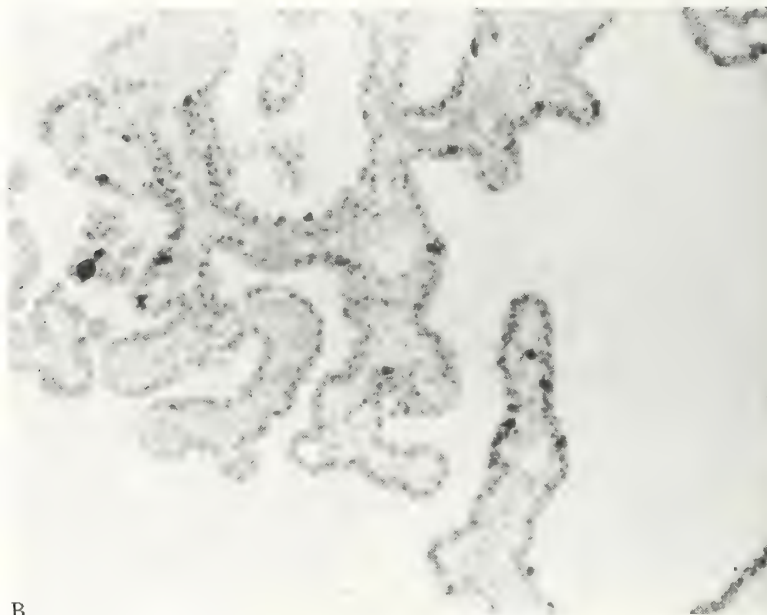
FIGURE 10.—Eyes from 2½-year-old Chediak-Higashi bull contrasted to the single eye from a normal Hereford.

cytes. The eosinophil from affected cattle resembles that from a horse, which normally has very large granules (fig. 12).

It is of considerable interest that an anomaly of this type results in an increased responsiveness to a chronic viral agent such as Aleutian disease of mink. In our paper, "Some observations on the natural occurrence of Aleutian disease," which is included in this symposium, there is ample evidence for the difference in susceptibility as measured experimentally by time of death



A



B

FIGURE 11.—A. Ciliary process from a normal Hereford. H. and E. $\times 700$.
B. Ciliary process from a Chediak-Higashi cow. H. and E. $\times 700$.

and by the mortality rate in naturally occurring Aleutian disease. In addition it is interesting that the difference does not occur when "fast" viral agents such as distemper or mink virus enteritis are involved or where a toxin such as botulism is used. The mechanism of the difference in susceptibility is unknown but it is possible that it is related to macrophage or eosinophil function.

Aleutian *aa* mink are much more susceptible to abscesses and to chronic bacterial infections of all types than *Aa* or *AA* mink. Among the partial albino cattle in our herd we do not have a particular problem with an agent such as Aleutian disease virus in mink. The average age of death of the albino animals in this herd is 12.2 months. All have died with multiple abscesses in the liver and lungs (see fig. 9). Bacteria

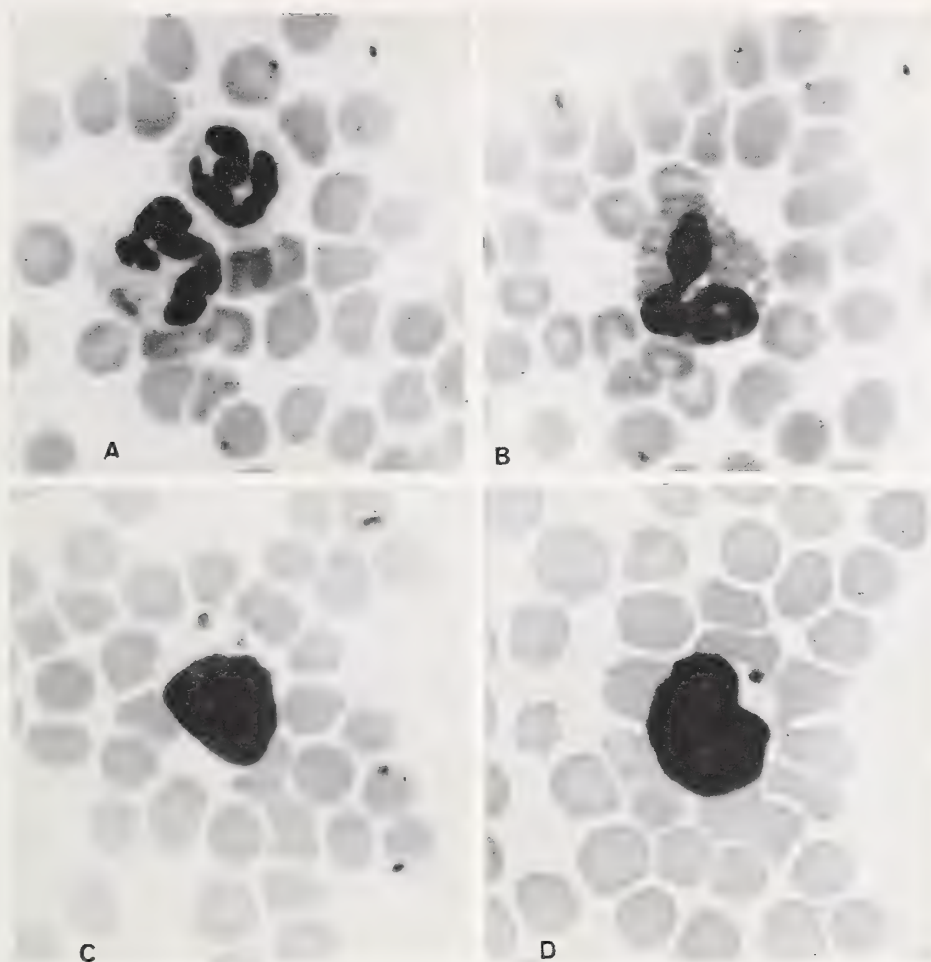


FIGURE 12.—Composite of bovine cells: (A) Polymorphonuclear leukocyte; (B) Eosinophil; (C) Lymphocyte; (D) Monocyte. These cells all show abnormal cytoplasmic granules. These are bovine cells, but the abnormality is of almost identical staining and morphologic character in children and mink (see fig. 2). Giemsa $\times 1300$.

of various kinds were isolated and there was no consistent pattern.

In mink and cattle there has been no lympho-reticulo-histiocytic infiltrate in the tissues such as occurs in nearly one-half of the cases of the Chediak-Higashi syndrome in man. If this infiltrate is caused by a specific agent it apparently does not attack mink or cattle.

SUMMARY

There is available in hereditary leukomelanopathy (Chediak-Higashi syndrome) of children and its analogues in other species a congenital anomaly which presents interesting experimental possibilities for studying:

(a) The ontogeny of melanin, by investigating the biochemical defect to determine where and at what stage in cell development it occurs,

(b) The origin and function of leukocyte granules or lysosomes in general. The defect may involve lysosomes in liver, pancreas and other organs, as it apparently affects neurons in addition to melanin pigmentation and leukocyte granulation, and

(c) Mechanisms of nonspecific resistance to bacterial and viral infection.

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Bibliography on Chediak-Higashi Syndrome

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Chediak-Higashi Syndrome

Synonyms:

In man:

- Anomalous panleukocytic granulations.
- Anomaly of Chediak.
- Béguez César-Chediak-Higashi syndrome.
- Béguez César-Steinbrinck-Chediak-Higashi syndrome.
- Chediak disease.
- Chediak-Higashi anomaly.
- Chediak-Hikashi disease.
- Chediak's anomaly of leukocytes.
- Chediak-Steinbrinck anomaly.
- Chediak-Steinbrinck disease.
- Chediak-Steinbrinck-Higashi syndrome.
- Congenital gigantism of peroxidase granules.
- Familial erythrophagocytic lymphohistiocytosis.
- Granulation anomaly of leukocytes.
- Hereditary-constitutional giant granulations of leukocytes.
- Hereditary leukomelanopathy.
- Leukocytic anomaly of Chediak.
- Panmyelopathy with Döhle bodies, thrombocytopenia and erythroblastosis (Hegglin syndrome).

In animals:

- Abnormal leukocyte bodies in mink.
- Albino cattle.
- Albino-dwarfism in cattle.
- Albinism in cattle.
- Albinotic dilution of color in cattle.
- Chediak-Higashi syndrome in mink and cattle.
- Hereditary abnormal leukocyte granules in mink.
- Lethal factor in grey Karakul sheep.

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Bibliography on Hemorrhagic Fevers in South America

Argentinian Hemorrhagic Fever

Bolivian Hemorrhagic Fever

Tacaribe Virus

compiled by

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Argentinian Hemorrhagic Fever:

Epidemic hemorrhagic fever in Argentina

Junin virus infection

O'Higgins disease

Stubble disease (mal de los rastrojos)

Hemorrhagic virosis of northwest Bonaerense

Epidemic hemorrhagic fever of Buenos Aires province

Endemo-epidemic febrile enanthematous leucopenia

Bolivian Hemorrhagic Fever:

Epidemic hemorrhagic fever in Bolivia

Machupo virus infection

Tacaribe virus infection

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A Note on Cinema of Slow Virus Infections

In keeping with the emphasis on the use of cinema in the Section for the Study of Child Growth and Development and Disease Patterns in Primitive Cultures and also the Laboratory on Slow, Latent, and Temperate Virus Infections it is felt that this monograph would be incomplete without some reference to the cinema of slow virus infections. The concept of the research film was outlined by E. R. Sorenson and D. C. Gajdusek, in "The Investigation of Nonrecurring Phenomena: The Research Cinema Film," *Nature*, 200, 112-114, 1963, and the full catalog and index of the research film made by the Section is being published in a Supplement to *Pediatrics* in January 1966.

At the Workshop, cinema taken for research and documentation purposes was presented on scrapie and on mink disease by a number of participants and the host section and laboratory presented edited films on scrapie infection in mice and on kuru. It is known that many other participants had made use of film in their research work and it is hoped that at such workshops in the future more opportunity can be given for describing and discussing work completed and work in progress through the medium of film, and thus provide incentive to make use of it, not only in the documentation of research, but also as a research tool in its own right.

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